A STUDY OF THE EARLY SCHWANN CELL LINEAGE: GROWTH FACTORS INVOLVED IN THE SURVIVAL AND MATURATION OF THE SCHWANN CELL PRECURSOR *IN VITRO*.

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ABSTRACT

Cells of the Schwann cell lineage are derived from the cells of the neural crest. Schwann cell development, including factors controlling myelination have been studied extensively, but little is known about the development of the glial cells found in early nerves. This work describes an intermediate cell type in the development of Schwann cells from the neural crest, the Schwann cell precursor.

Using immunocytochemistry, the molecular phenotype of these cells has been investigated. A study has been made of S100 expression and survival *in vitro* in defined medium, on cells isolated from E14 to newborn nerves. These experiments show that glial cells isolated from E14-E15 nerves are not neural crest cells. The transition from precursor to Schwann cell occurs rapidly between E15 and E17 *in vivo*.

Death of precursors in defined medium *in vitro* has been examined using DNA staining and studies of DNA fragmentation. The results indicate that death is by apoptosis, which can be delayed by addition of protein synthesis inhibitors or elevation of cyclic AMP (cAMP). Culture in neurone-conditioned medium suppresses apoptosis.

A large number of growth factors have been investigated for survival effects. Three members of the fibroblast growth factor family have been found to promote short-term precursor survival when used in combination with a member of the insulin growth factor family. An extensive investigation has been made of the combinations required for precursor survival, both in the presence and absence of elevated cAMP.

Two families of growth factors, Neu differentiation factors (NDFs) and endothelins are shown to promote long-term survival of the precursors. Precursor maturation in these factors has been followed using immunocytochemistry and assays for survival and proliferation. NDFβ-2, but not endothelin, promotes maturation of precursors to Schwann cells with a time course similar to that seen *in vivo*. Addition of endothelin in the presence of NDFβ-2 delays maturation. These results indicate that there is likely to be a complex interplay of growth factors controlling Schwann cell precursor survival and generation of Schwann cells during nerve development.

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ABBREVIATIONS

5-HT - 5-hydroxytryptamine

α-MSH - α melanocyte stimulating hormone

aFGF, FGF-1 - acidic fibroblast growth factor

AraC - cytosine arabinoside

ARIA - acetylcholine receptor-inducing factor

BDNF - brain-derived growth factor

bFGF, FGF-2 - basic fibroblast growth factor

bHLH - basic-helix-loop-helix

BMP - bone morphogenetic protein

BrdU - bromodeoxyuridine

BSA - bovine serum albumin

CMT - Charcot-Marie-Tooth disease

CNP - 2', 3'-cyclic nucleotide 3'-phosphdiesterase

CNS - central nervous system

CNTF - ciliary neurotrophic factor

CS - calf serum

CSF - colony stimulating factor

Cyclic AMP, cAMP - adenosine 3',5'-cyclic monophosphate

DAG - 1,2-diacylglycerol

DiI - 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

DM - defined medium

DMEM - Dulbecco's modified Eagle's medium

DNA - deoxyribonucleic acid

DRG - dorsal root ganglia

E - embryonic day

EC_{50} - half-maximal effective concentration

ECM - extracellular matrix

EDTA - ethylenediaminetetraacetic acid

EGF - epidermal growth factor

ENS - enteric nervous system

ET - endothelin

FCS - foetal calf serum

GalC - galactocerebroside

GABA - γ-aminobutyric acid

GAP-43 - growth associated protein

GDNF - glial-derived neurotrophic factor

GFAP - glial fibrillary acidic protein

GGF - glial growth factor

Hepes - N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

HS - horse serum

HSPG - heparan sulfate proteoglycan

ICE - interleukin-1β-converting enzyme

Ig - immunoglobulin

IGF - insulin-like growth factor

IGFBP - insulin-like growth factor binding protein

kD - kilodalton

K-FGF, FGF-4 - Kaposi's sarcoma fibroblast growth factor

LIF - leukaemia inhibitory factor

MAG - myelin associated protein

MBP - myelin basic protein

MEM - modified Eagle's medium

MHC - major histocompatibility complex

mRNA - messanger ribonucleic acid

MTT - 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

N-CAM - neural cell adhesion molecule

NCM - neurone conditioned medium

NDF - neu differentiation factor

NGF - nerve growth factor

NMJ - neuromuscular junctions

NT-3 - neurotrophin-3

NT-4 - neurotrophin-4

p75LNGFr - p75 low affinity nerve growth factor receptor

P - post-natal day

PARP - poly (ADP-ribose) polymerase

PBS - phosphate buffered saline

PDGF - platelet-derived growth factor

PKC - protein kinase C

PLL - poly-L-lysine

PLP - proteolipid protein

PMP22 - peripheral myelin protein, 22kD.

PNS - peripheral nervous system

RA - retinoic acid

RNA - ribonucleic acid

RT- room temperature

SA - sympathoadrenal

SCF - stem cell factor, Steel factor, mast cell growth factor, kit ligand

SD - standard deviation

SDS - sodium dodecyl sulphate

SEM - standard error of the mean

SMP - Schwann cell myelin protein

SP - substance P

TGFβ - transforming growth factor β

TNF - tumour necrosis factor

TPA - 12-O-tetradecanoylphorbol-13-acetate

VIP - vasoactive intestinal peptide

CHAPTER 1

GENERAL INTRODUCTION

The glial cells of the peripheral nervous system

Glia are the cells of the nervous system that surround and support the neurones and their axons. Originally their function was believed to be purely structural, acting as a glue to hold the nerve tissue together, but a century and a half of research has revealed that they have important functions within the nervous system. In the peripheral nervous system (PNS), the neurones and glia are mainly derived from the transient population of the neural crest. These rapidly migrating cells populate the sensory and autonomic ganglia and the peripheral nerves, as well as contributing to other cell types that will be described in detail later on. The glial cells that are derived from the neural crest fall into four distinct types that will be described below. Unless stated otherwise, the antigenic phenotype described is that of the rat.

Satellite cells

Satellite cells are the major glial cells found in sensory and autonomic ganglia other than those of the enteric nervous system (see below). Many of these cells together ensheath the neuronal cell bodies within the ganglia. This cover is continuous and in some places may consist of several overlapping processes of the satellite cells (Pannese, 1960; 1968). During development of the ganglia, these cells differentiate after the neurones and their numbers are closely regulated to the number and size of the neuronal cell bodies within the ganglia. After neuronal differentiation, the neurones no longer divide but the volume of each cell body increases, expanding the area of the neuronal cell membrane. Satellite cells undergo mitosis even after they have differentiated in terms of position and morphology. This increase in number is directly correlated with the increase in the area of the neuronal perikaryon. Thus, the total ensheathment of the neurones is maintained during development (Pannese, 1968; Hall and Landis, 1991; 1992). Basal lamina made by the satellite cells covers the glial cells and ensheathed neurones separating them from other elements of the extracellular matrix that are present in both sensory and sympathetic ganglia (Pannese, 1968; Baluk et al, 1985; Bannerman et al., 1986).

The satellite cell-neuronal association is not static; when cells of a mouse parasympathetic ganglion were studied *in vivo* over several weeks the positions of the

satellite cells relative to the neuronal cell bodies changed (Pomeroy and Purves, 1988). Preganglionic synaptic terminals were found to be more prevalent in the regions of the neurone closest to the satellite cell nucleus. It was suggested that the changes in satellite cell position were associated with synaptic remodelling. Satellite cells of dorsal root sensory ganglia (DRG) of adult rats have been shown to respond to axotomy or a localized inflammatory reaction by proliferating. The response of the satellite cells is rapid, with the peak of proliferation occuring during the first week after injury (Lu and Richardson, 1991).

Satellite cells from both sympathetic and sensory ganglia express some proteins found in common with all peripheral glia (shown in Table 1.1). In addition to these proteins, four monoclonal antibodies, GL1, GL2, G1 and G2, raised against undifferentiated embryonic chick DRG, show strong expression in sensory ganglion satellite cells but are absent from sympathetic ganglion satellite cells. Culture of sympathetic ganglion satellite cells resulted in the cells acquiring expression of all four epitopes, suggesting that lack of expression in vivo is due to environmental factors (Rüdel and Rohrer, 1992).

Enteric glia

The enteric nervous system (ENS) is unlike other areas of the PNS, and in structure more closely resembles the central nervous system (CNS). It contains a large number of neurones, calculated to be comparable to the number of neurones present in the spinal cord. These neurones are organised into two interconnected plexuses, the outer myenteric plexus and the inner submucosal plexus (reviewed by Gabella, 1979). Sensory-, motor- and inter-neurones are all represented within the ENS and the majority of the neurones do not receive extrinsic input from the CNS. Surrounding these neurones are the enteric glia. These cells have an irregular morphology, and have been compared to the astrocytes of the CNS.

The enteric glia exhibit extensive branching and irregular processes which mingle with the neuronal processes forming a dense neuropil. They form a sheath around the neurones and axons of the enteric plexuses. The sheath is incomplete, however, and both axonal varicosities and neuronal cell bodies extend through the sheath in places, contacting the extracellular connective tissue (reviewed in Gershon and Rothman,

1991). The association of enteric glia with axons involves communal ensheathment of axon bundles. Since individual glial-neuronal units are not formed in enteric ganglia, basal lamina is found covering the whole enteric ganglion rather than individual enteric glia (Bannerman et al., 1986); elements of the basal lamina and extracellular matrix are not found within the enteric ganglia.

Absence of fibrillar collagen within the enteric ganglia and the presence of high concentrations of the intermediate filament glial fibrillary acidic protein (GFAP) within the enteric glia (also found in large amounts in astrocytes of the CNS, as well as in Schwann cells) has suggested to some authors that they play a major role in structural support of the ENS (reviewed in Gershon and Rothman, 1991, Jessen and Mirsky, 1980). Another morphological feature that is comparable to the environment of the CNS is the absence of blood capillaries within the nerve plexuses.

When placed in culture, guinea pig enteric glia have a higher basal level of proliferation than Schwann cells under the same conditions (Eccleston et al., 1987a). When cultured with enteric neurones, however, the level of proliferation is reduced, suggesting that enteric neurones exhibit an inhibitory effect on enteric glial division (Eccleston et al., 1989).

In common with other glial cells of the PNS, enteric glia express S100 (Bannerman et al.,1988) but, unlike most other non-myelinating glial cells, they do not express the low affinity nerve growth factor receptor (p75LNGFr) (Bannerman et al., 1986, 1988). Expression of p75LNGFr is upregulated in cultures of rat enteric glia indicating that suppression is regulated by the enteric environment (Jessen and Mirsky, 1983). Another molecule that displays this property is the avian Schwann cell myelin protein (SMP). Expressed on all avian Schwann cells, it is absent from enteric glia and satellite cells *in vivo* (Le Douarin and Ziller, 1993), but culture of these cells leads to SMP expression, showing that repressive elements found in the ENS or peripheral ganglia control some aspects of the glial phenotype.

Some of the antigens expressed by non-myelin-forming Schwann cells are also found on rat enteric glia (see Table 1.1), but unlike non-myelin-forming Schwann cells, enteric glia, *in vivo* and *in vitro* do not express the myelin-associated glycolipid, galactocerebroside (GalC) (Jessen and Mirsky, 1983) or the growth-associated protein

GAP-43 (Stewart et al., 1992). Unlike Schwann cells, enteric glia express cell surface gangliosides recognised by the antibodies A2B5 (100% of cells) and LB1 (on 95% of cells) (Bannerman et al., 1988).

Teloglia / perisynaptic Schwann cells

Teloglia are the specialised Schwann cells found surrounding the neuromuscular junctions (NMJ) where they appear to play an important role in maintenance and regeneration of NMJs. These cells send out fine processes enclosing the nerve terminal to within 50nm of the sites of neurotransmitter release (Georgiou et al., 1994).

These cells respond to secretion of neurotransmitters by elevating intracellular levels of Ca^{2+} and are thus active participants during synaptic transmission (Jahromi et al., 1992). Myelin-forming Schwann cells, in the last segment of nerve before the NMJ, showed no changes in Ca^{2+} levels under the same conditions suggesting that they do not express receptors for the neurotransmitters. There is evidence that both muscarinic and purinergic receptors are present on the teloglia (Georgiou et al., 1994; Robitaille, 1995). The functional consequence of the rise in teloglial intracellular Ca^{2+} is unknown, but it is possible that it has a role in maintenance of the teloglial phenotype (Georgiou et al., 1994).

Unlike non-myelin-forming Schwann cells in other areas of the peripheral nerves, these cells do not normally express GFAP or the growth associated protein GAP-43 (Georgiou, 1994; Woolf et al., 1992). If the motor endplate is denervated, teloglia elaborate extensive processes beyond the NMJ, which are retracted on reinnervation (Reynolds and Woolf, 1992). Denervation or blockade of nerve activity upregulates the expression of GFAP and GAP-43 in these cells (Woolf et al, 1992; Georgiou et al., 1994). Inhibition of GFAP expression in normal teloglia appears to be brought about by release of neurotransmitters from the nerve terminal: exogenous muscarine has been shown to reduce the number of teloglia expressing GFAP in denervated NMJ. It is possible that the increase in teloglial intracellular Ca^{2+} plays a role in inhibiting GFAP expression (Georgiou et al., 1994).

The processes elaborated by teloglia after denervation appear to guide regrowing axons to the denervated endplates, and in cases of partial denervation, to guide sprouts

from neighbouring innervated endplates to form endplates on the denervated muscle (reviewed in Son et al., 1996).

Schwann cells

Schwann cells are the glial cells that ensheath axons of peripheral nerves. Two differentiated types of Schwann cell exist in the adult nerve: non-myelin-forming Schwann cells that ensheath numerous small diameter axons, and myelin-foming Schwann cells that ensheath a single large diameter axon. Both cell types display characteristic morphologies and molecular phenotypes that will be described below. They are derived from a common progenitor cell that populates the nerve before birth. When cells of either type are removed from axonal contact and cultured *in vitro*, they revert to a cell with a protein phenotype similar to the Schwann cells found in embryonic nerves prior to differentiation (Table 1.1; Jessen and Mirsky, 1991). These de-differentiated cells can be made to re-express markers of either phenotype given the correct culture conditions or by reassociation with axons (Jessen and Mirsky, 1991; Guénard et al., 1995).

Non-myelin-forming Schwann cells

Non-myelinated nerve fibres, first observed by Remak in 1838, are covered by a continuous row of non-myelin-forming Schwann cells. Each of these cells ensheaths many small diameter axons. The axons either lie in troughs of Schwann cell cytoplasm or are entirely wrapped by thin processes cytoplasm, preventing contact between axons (reviewed by Thomas and Ochoa, 1984). The axon bundles within the non-myelin-forming Schwann cell change along the length of the cell: a Schwann cell may ensheath a particular group of axons at its proximal end but ensheath a different group at its distal end. In the places where the axons are incompletely wrapped they are separated from the surrounding extracellular matrix of the endoneurium by the basal lamina made by the Schwann cells. This basal lamina is continuous along the length of the non-myelinated fibre (Gould et al, 1992).

Some of the antigens expressed by the non-myelin-forming Schwann cells in the rat are shown in Table 1.1. In common with the other glial cells of the PNS, non-myelin-forming Schwann cells express S100, (Holton and Weston, 1982a). The glycosphingolipid galactocerebroside (GalC) is a major component of the myelin

sheath but is also found in the membrane of the non-myelin-forming Schwann cell (Jessen et al., 1985; Eccleston et al, 1987b). Loss of axonal contact downregulates the expression of this lipid, but re-association of these cells with axons leads to its re-expression (Jessen et al., 1987a, 1987b). Other lipid antigens that are associated with both non-myelin-forming and myelin-forming Schwann cells are 04, 08 and 09. The expression of the 04 antigen on Schwann cells precedes the differentiation of the two mature Schwann cell phenotypes (see below; Mirsky et al., 1990), while 08 and 09 antigens are not expressed on the majority of non-myelin-forming Schwann cells until several weeks after they are found on myelin-forming Schwann cells. Expression of these lipid antigens is also regulated by axonal contact (Eccleston et al., 1987b).

Myelin-forming Schwann cells

The myelin-forming Schwann cell is associated with a single, large diameter axon, elaborating a spiralling sheath around the axon (Peters and Muir, 1959; reviewed in Thomas and Ochoa, 1984). Each Schwann cell-axon unit is referred to as an internode, each internode is separated from the next by the node of Ranvier, a region of the axon not covered by myelin but by finger-like processes of Schwann cell cytoplasm forming the nodal collar (Thomas and Ochoa, 1984). The internodes of a given axon are of similar dimensions, with the distance between each node of Ranvier and the thickness of the myelin sheath being remarkably constant, both being proportional to the diameter of the axon (reviewed in Bray et al., 1981). The whole myelinated fibre, including the nodes of Ranvier is covered by a continuous basal lamina produced by the Schwann cells (Gould et al., 1992).

The Schwann cell myelin sheath is a remarkable specialisation of the cell membrane. During myelin formation, the membrane expands and wraps spirally around the axon forming the multilamellar sheath (Bunge et al., 1989). Compaction of the myelin follows with extrusion of most of the cytoplasm from the myelin sheath. Cytoplasm is found in specific regions of the Schwann cell. It is found in the region of the nucleus which is situated on the outside of the sheath, and also in the innermost section of the sheath adjacent to the axon: the adaxonal cytoplasm. Narrow clefts of cytoplasm, the Schmidt-Lanterman incisures, traverse the myelin from the outer to the inner surfaces, and extensions of cytoplasm are found at the edges of the Schwann cells at the nodes of Ranvier, the perinodal loops (Thomas and Ochoa, 1984).

The membrane composition is lipid-rich which enables the myelin sheath to act as an insulator around the nerve. In the electron microscope, a cross-section of myelin appears as a series of concentric circles. The intraperiod line is formed where the external surfaces of the plasma membrane are apposed, and the major dense line is formed by apposed cytoplasmic surfaces of the membrane. A high density of sodium channels are present in the axonal membrane at the node which, on depolarization, allows an inward current in this region (Dugandzija-Novakovic et al., 1995). The depolarization 'jumps' from node to node, and results in rapid conduction of the nerve impulse; this is known as saltatory conduction (Bray et al., 1981).

The molecular phenotype of the myelin-forming Schwann cell reflects the specialization of the membrane; many proteins are found exclusively in these cells or are restricted to myelin-forming Schwann cells and oligodendrocytes, the myelin-forming cells of the CNS. In contrast, many proteins found on other glial cells of the PNS are absent from or expressed at very low levels in myelin-forming cells (Table 1.1). In most cases, the expression of these proteins is rapidly upregulated if the cells are removed from axonal contact (Mirsky and Jessen, 1990), but GAP-43 shows a longer time course for re-expression (Curtis et al., 1992). The Ca^{2+}- binding protein S100 is present in myelin-forming Schwann cells and the level of expression is higher than that found in non-myelin-forming Schwann cells (Holton and Weston, 1982a; Mata et al., 1990). As stated earlier, GalC, the lipid antigens 08 and 09, as well as 04 and sulfatide A007 are expressed by myelin-forming Schwann cells, but expression is rapidly down-regulated if the cells are removed from axonal contact (Jessen et al., 1985, 1987a; Eccleston et al., 1987b; Mirsky et al., 1990).

Similarly, other proteins and lipids associated with the mature myelin-forming phenotype are lost if the cell is removed from axonal contact. Major myelin proteins expressed by the cells are P_0, myelin basic protein (MBP), the cell adhesion molecule myelin associated protein (MAG), peripheral myelin protein (PMP) 22, and the small basic protein P_2 (Mirsky et al., 1980; Trapp et al., 1981, 1984, 1988; Winter et al., 1982; Trapp and Quarles, 1984; Lemke and Axel, 1985; Griffiths et al., 1989; Pedraza, et al., 1991; Welcher et al., 1991).

P_0 is the major protein in PNS myelin, accounting for 50% of the total protein (reviewed by Hudson, 1990). It is a glycosylated protein with an extracellular domain

homologous to the Ig superfamily. Evidence suggests that it functions as a homophilic adhesion molecule, holding the wrapped membranes together in the myelin sheath (D'Urso et al., 1990; Filbin et al., 1990; Filbin and Tennekoon, 1993). In mutant mice lacking P_0, the axons are hypomyelinated with varying levels of myelination; some axons are myelinated, and a few exhibit quite thick myelin sheaths, but there is a lack of compaction; other large diameter axons are ensheathed by Schwann cells but lack any myelin spirals (Giese et al., 1992). Point mutations in the human P_0 protein are associated with the hereditary sensory neuropathy Charcot-Marie-Tooth (CMT) type 1B disease (Hayasaka et al., 1993).

Although associated with myelin-formation, the mRNA for P_0 is expressed very early in nerve development, and in migrating neural crest cells in both chick and rat (Bhattacharyya et al., 1991; Lee, Brennan et al., in preparation). Low levels of protein expression can be detected early in rat development and these continue to be expressed on non-myelin-forming Schwann cells even in the adult (Lee, Brennan et al., in preparation). Recently, a case has been made for high level P_0 expression being the developmental default state for Schwann cells, with active down-regulation of P_0 protein prior to myelination by axonal signals (Cheng and Mudge, 1996). However, it appears that the experiments have used sensitive detection of basal levels of P_0 protein, and evidence from this laboratory has shown that high levels of P_0 protein found in myelinating Schwann cells are not detected in culture, and high levels of mRNA for P_0, such as those associated with myelination, are down-regulated in the absence of axonal contact (Lee, Brennan et al., in preparation).

Proteolipid protein, (PLP) and the alternatively spliced variant DM20 are expressed by Schwann cells but are not incorporated into myelin. This is in contrast to the CNS where PLP is one of the most abundant myelin proteins, constituting 50% of the membrane protein of oligodendrocytes (reviewed by Mikoshiba et al., 1991). Expression of PLP mRNA in the PNS is not regulated by axonal contact, unlike the other myelin proteins (Gupta et al., 1991).

PMP22 is an integral membrane protein expressed mainly by myelin-forming Schwann cells, (Snipes et al., 1992). It has been found to be homologous to the growth arrest-specific gene Gas-3, one of a group of genes of unknown function found in quiescent cells (Welcher et al., 1991). Duplication of the human PMP22 gene

results in CMT type 1A disease. Rats over-expressing PMP22 exhibit symptoms similar to that seen in CMT1A, with PNS hypomyelination, Schwann cell hypertrophy and muscle weakness indicating that this protein is important in normal PNS myelination (Sereda et al., 1996). It has been suggested that PMP22 and PLP/DM20 play analogous roles in the PNS and CNS respectively (reviewed in Snipes et al., 1993).

MBP is another structural protein found in myelin with many isoforms due to alternate splicing (reviewed in Hudson, 1990; Mikoshiba et al., 1991). MBP is a component of the major dense line of the myelin sheath (Omlin et al., 1982) but is expressed in the PNS at much lower levels than that seen in the CNS (Hudson, 1990). It is not essential for normal myelin sheath formation in the PNS, but in mutant mice that lack MBP (*shiverer*) there is an increase in the number of Schmidt-Lanterman incisures (Gould et al., 1995).

MAG is a heavily glycosylated protein that is found in myelin-forming Schwann cells on the periaxonal membrane and non-compacted surfaces (reviewed in Colman, 1991). It is a member of the immunoglobulin superfamily and, like P_0, has adhesive properties. It is localized at the axon-Schwann cell interface and first appears at the start of myelination, (reviewed in Jessen and Mirsky, 1993). In mice lacking MAG, myelin sheaths are essentially normal, but some nerves exhibit an absence of cytoplasm from the periaxonal collar and separation of the axon from the surrounding sheath (Li et al., 1994). These results indicate that MAG is important for the adhesion or spacing of myelin in the periaxonal region.

P_2 is found in compact myelin in a proportion of the myelin-forming cells, mainly those with thicker myelin sheaths (Winter et al., 1982). It has been cloned in mouse, rabbit and human and shown to belong to the family of fatty acid binding proteins (Narayanan et al., 1988, 1991, 1994). It is thought to be involved with assembly and maintenance of the myelin lipids (Gould et al., 1992). In the same manner, the lipid antigen 011 is expressed on some myelin-forming Schwann cells that have thicker myelin sheaths (Eccleston et al., 1987b); it is not clear whether P_2 and 011 are present on the same population of Schwann cells.

Expression of the enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) in uncompacted myelin and colocalization of this molecule with the cytoskeleton have suggested that CNP is involved in myelin assembly (Hudson, 1990). However, it is found on all Schwann cells in culture and is therefore likely to be expressed by non-myelin-forming Schwann cells (Jessen and Mirsky, 1993).

Periaxin (also known as Schwann cell membrane glycoprotein (SAG; Dieperink et al., 1992) and p170k (Shuman et al., 1983)) is a membrane protein found in myelin-forming Schwann cells. It is thought to be involved in axon-glial interactions and in the early stages of myelin formation (Gillespie et al., 1994). This protein is found in the first uncompacted layers of Schwann cell membrane that ensheath the axon at the start of myelination. Subsequently, it is excluded from compacted myelin and is found in the periaxonal cytoplasm, where it colocalizes with MAG. It is thought to have a role in cytoskeletal interactions during myelination, possibly involving interactions with MAG (Gillespie et al., 1994; Scherer et al., 1996).

Appearance of Schwann cell antigens in development

The antigenic phenotype described above and in Table 1.1 is that of the mature myelin- and non-myelin-forming Schwann cells. Just prior to birth and the start of myelination, the cells exhibit a fairly uniform phenotype, with expression of antigens that are associated more with the mature non-myelin-forming Schwann cell. A diagrammatic representation of Schwann cell development is given in Fig. 1.1. The Ca^{2+}-binding protein S100 that is found in all mature peripheral glia, is first seen in Schwann cells from E16-17 nerves and will be discussed in detail in Chapter 3. The lipid antigen 04 is first apparent in $S100^{+}$ cells from E16, this increases to 40% of the cells at E18 and it is expressed by essentially all Schwann cells at E20 (Mirsky et al., 1990). Interestingly, 04 is thought to be the sulphated form of GalC which appears two days later in development (Mirsky et al., 1990) and is first detected at E19 in cells that will become myelin-forming Schwann cells. It is possible that the antibody to 04 detects antigens other that sulphated GalC (R. Mirsky, personal communication). Expression of GalC is also seen in non-myelin-forming Schwann cells but appears at a later stage in development, in the third postnatal week (Jessen et al., 1985).

GFAP expression starts to appear at E18 in both myelin- and non-myelin-forming Schwann cells but its expression is only transient in myelin-forming cells, being downregulated soon after myelination starts. It continues to be expressed in non-myelin-forming Schwann cells (Jessen et al., 1990).

Most recently antibodies recognizing the CD9 antigen on platelets have been found to recognize an antigen present on Schwann cells (Hadjiargyrou and Patterson, 1995; Kaprelian et al., 1995; Anton et al., 1995). The CD9 antigen is a tetraspan protein with similar tertiary structure to PMP22, PLP and gap junction connexins (Mirsky and Jessen, 1996). Antibodies to this antigen enhance Schwann cell adhesion and migration on DRG neurites (Anton et al., 1995; Hadjiargyrou and Patterson, 1995). The CD9 antigen is present on rat Schwann cells from E17 (Tole and Patterson, 1995; Anton et al., 1995).

Schwann cells during nerve degeneration

Following nerve injury that results in severence of axons, Schwann cells distal to the point of injury undergo extensive changes with loss of myelin and massive proliferation of the Schwann cells in this region of the nerve. The sequence of events following axotomy is referred to as Wallerian degeneration (reviewed in Fawcett and Keynes, 1990; Scherer and Salzer, 1996). It is not known what triggers the response of the Schwann cells to axotomy. Products released by the damaged axons or invading macrophages might promote demyelination and Schwann cell proliferation, in addition to changes caused by interruption of axonal signals required for maintenance of the mature Schwann cell phenotype (Biachwal et al., 1988; Oaklander and Spencer, 1988; Hall, 1989; Perry and Brown, 1992; Scherer and Salzer, 1996).

Following loss of axonal contact, myelin is actively broken down in the distal region of the damaged nerve. Clearance of the axonal and myelin debris is performed by Schwann cells and macrophages (Beuche and Friede, 1984; Bigbee et al., 1987; Stoll et al., 1989; Brown et al., 1991). Schwann cells can engulf and degrade the debris in the absence of macrophages (Fernandez-Valle et al., 1995), but their presence enhances the response of the Schwann cells (Clemence et al., 1989; Fernandez-Valle et al., 1995).

Both myelin- and non-myelin-forming Schwann cells undergo a period of rapid division with a peak 3-5 days post-axotomy (Oaklander and Spencer, 1988; Clemence et al., 1989;). The tubes of basal lamina around the Schwann cells are not broken down and the proliferating cells are retained by them, forming the bands of Büngner (Scherer and Salzer, 1996).

During this period of degeneration, while myelin proteins are lost from myelin-forming Schwann cells, and the lipids GalC and 04 are lost from both types of Schwann cell (reviewed in Gould et al., 1992), antigens found on the non-myelin-forming Schwann cells are re-expressed by the previously myelinating cells. Expression of NCAM, L1, GFAP, A5E3, Ran-2 and p75LNGFr is rapidly upregulated in de-differentiated cells, harking back to the phenotype found in the nerve prior to myelination (Gould et al., 1992). GAP 43, however, is upregulated 4-8 weeks after injury indicating that regulation of its expression is controlled differently from these proteins (Curtis et al., 1992).

Since L1 and NCAM are known to play a major role in promoting axonal outgrowth, the upregulation of these molecules in damaged nerves is likely to be significant for nerve repair (reviewed in Schachner, 1990). Axonal growth into intact nerves is extremely limited suggesting that the loss of mature Schwann cell phenotype is essential for the regeneration of nerves (Brown et al., 1991). Further evidence for this comes from cultures of adult rat DRG neurones which will extend neurites on a substrate of predegenerated sciatic nerve but fail to grow on intact nerves (Bedi et al., 1992). The presence of Schwann cells appears to be necessary for successful axon regrowth; in acellular grafts containing only basal lamina, axons grow from the proximal stumps of the cut nerve but for limited distances and in the presence of accompanying Schwann cells from the proximal stump (reviewed in Fawcett and Keynes, 1990; Ide et al., 1983; Feneley et al., 1991). Where Schwann cell migration is prevented, little or no axonal outgrowth is observed (Hall, 1986).

In addition to providing a suitable substrate for axon regeneration, Schwann cells are believed to be a source of survival factors for neurones after nerve lesion. The mRNA for the neurotrophic factors NGF and brain-derived neurotrophic factor (BDNF) and for the neurokine LIF are upregulated in Schwann cells after axotomy (reviewed in Scherer and Salzer, 1996; Heumann et al., 1987a, 1987b; Meyer et al., 1992; Curtis et

al., 1994). These factors are released from the cells and promote neurite sprouting and outgrowth as well as acting as survival factors for axotomised neurones (Heumann et al., 1987a, 1987b; Acheson et al., 1991; Freidman et al., 1992; Rende et al, 1992; Meyer et al., 1992). CNTF is also produced by Schwann cells, but the mRNA expression in the distal stump decreases following nerve lesion, although biological activity of CNTF is detectable in the distal region for several days after injury (Sendtner et al., 1992). As yet, production of NT-3 or NT4/5 has not been reported from Schwann cells. Both the neurotrophins and the neurokines can be retrogradely transported to the neuronal cell bodies following release from the Schwann cells where they can enhance the survival of regenerating neurones (Curtis et al., 1993, 1994, 1995; Scherer and Salzer, 1996).

Reinnervation of the nerve distal to the point of injury involves regrowth of the axons into the basal lamina tubes containing the Schwann cells. Association of the Schwann cells and axons is similar to that seen during late embryonic development, with Schwann cells initially surrounding bundles of axons. Eventually, Schwann cells form a 1:1 relationship with the larger diameter axons and start to form myelin. However, in these regenerated nerves, the thickness of the myelin sheath is never as great as in undamaged nerves, and the length of the internodes are shorter (reviewed in Scherer and Salzer, 1996).

Other Schwann cell functions

As well as enabling rapid nerve impulse conduction, Schwann cells have been reported to perform other important functions in the PNS. Schwann cells actively influence the axons they ensheath. Myelination increases the caliber of axons apparently by increasing the phosphorylation of neurofilaments within the axons (de Waegh et al., 1992). The presence of myelin also affects the slow transport mechnism within axons. In Trembler mice, a point mutation in the PMP22 protein results in dysmyelination, and peripheral axons in these mice exhibit increased density of neurofilament and decreased slow axonal transport (de Waegh and Brady, 1990; de Waegh et al., 1992; Suter et al., 1992). Schwann cells also control the distribution of ion channels in the axolemma, restricting their expression to the paranodal regions. Following demyelination, sodium channel expression in the axonal membrane becomes diffuse. Subsequent distribution of sodium channels is controlled by the presence of Schwann

cells, with clusters of sodium channels becoming focused at the new nodes of Ranvier after re-myelination is complete (Dugandzija-Novakovic et al, 1995).

Schwann cells exhibit ion channels both *in vivo* and *in vitro* and neurotransmitter receptors *in vitro* (reviewed in Bevan, 1990; Barres et al, 1990). Several distinct ion channels selective for potassium ions have been described both in vivo and in vitro (Bevan, 1990; Mi et al., 1995). One of the channels described by Mi et al (1995) is selectively distributed in the myelin-forming Schwann cell membrane in the outer cytoplasmic loop and in the paranodal loops at the nodes of Ranvier. The localization of this type of channel would agree with one of the proposed functions of Schwann cells, that of buffering the K^+ levels around the axon following axon stimulation. Chloride ion and sodium ion channels are also found in Schwann cells although the latter is not found in myelin-forming Schwann cells (Bevan, 1990).

The expression of neurotransmitter receptors in Schwann cells has not been demonstrated in vivo. *In vitro*, however, they express β_1-adrenergic, vasoactive intestinal peptide (VIP), and serotonin receptors. Binding of ligands to these receptors produces a transient elevation of intracellular cyclic AMP (Yasuda et al., 1988; Yoder et al., 1996).). Purinergic receptor expression has also been reported in Schwann cells, and the expression appears to be controlled by axonal contact (Jahromi et al., 1992; Lyons et al., 1994, 1995).

Schwann cells can interact with cells of the immune system. The major histocompatibility complex (MHC) class II antigens are involved in the presentation of foreign antigens to lymphocytes and in initiating an immune response. Schwann cells can be made to express these antigens in response to exogenous interferon-γ or in the presence of mycobacterial antigens and T cells, suggesting that they may play a role in the immune response to mycobacterial infection (Samuel et al., 1987; Kingston et al., 1989).

Glial cells of the vertebrate central nervous system.

Four main classes of glia are found in the CNS: astrocytes, oligodendrocytes, microglia and ependymal cells. Of these, astrocytes and oligodendrocytes play major structural and functional roles within the CNS. The other two classes of cell have more restricted roles: microglia, derived from blood monocytes early in development,

act as macrophages within the CNS, and ependymal cells line the internal cavities of the CNS.

Oligodendrocytes are the myelin-forming cells of the CNS. However, unlike Schwann cells of the PNS, each oligodendrocyte can myelinate many axons (reviewed in Hudson, 1990). Many myelin proteins expressed by Schwann cells are also expressed by oligodendrocytes, but there are also major differences in expression some proteins, possibly related to the levels of compaction found in the different myelin sheaths; CNS myelin exhibits greater compaction than PNS. For example, the proteins P_0 and PMP22, involved in the compaction of PNS myelin are absent from oligodendrocytes, however, PLP, present as a cytoplasmic component within Schwann cells, is a major component of CNS myelin and functions in compaction of myelin (Hudson, 1990; Snipes et al., 1993). MBP is also expressed in different amount in the two systems, making up 30% of the CNS myelin proteins but only 5-15% of PNS myelin; again this protein is implicated in myelin compaction (Hudson, 1990).

Astrocytes are intimately associated with neurones and their processes throughout the CNS. During development, astrocytes act as guides for the migration of some neurones, providing a scaffold along which neurones can move (reviewed in Hatten et al., 1990). These cells contain large quantities of the intermediate filament GFAP, and are thought to provide structural support for the CNS which, unlike the PNS, lacks the mechanical support of collagen fibres. Astrocytes in the vicinity of blood vessels extend end feet that contact and surround the entire external surface of the vessels. By an as yet unknown method, these cells induce the endothelial cells lining the blood vessels to form tight junctions, forming the blood-brain barrier that restricts the transport of molecules into the brain (reviewed in Rubin, 1992; Schlosshauer, 1993). Astrocytes also express ion channels and are thought to play a major role in buffering the ionic environment (reviewed in Bevan, 1990). They also express neurotransmitter receptors, and are thought to control levels of neurotransmitters such as γ-aminobutyric acid (GABA), serotonin and glutamate by uptake from the external environment (Bevan, 1990).

Astrocytes are not uniform throughout the CNS; cells with different morphology, molecular phenotype and function have been found in different areas (reviewed in Wilkin et al., 1990; Barres et al., 1990). Extensive studies of the glial cells in the

developing optic nerve have been made which have revealed that *in vitro*, three glial cell types can be distinguished, astrocytes type 1 and 2, and oligodendrocytes (reviewed in Raff, 1989; Richardson et al., 1990). The type 1 astrocyte forms a fibroblast-like cell in culture and does not express the antigen detected by the antibody A2B5. This type of astrocyte can be found *in situ* in the optic nerve. This astrocyte develops from a separate lineage to the type 2 astrocyte, which is found as a process-bearing, $A2B5^+$ cell *in vitro*. Whether this latter cell is present *in vivo* has not been clarified (Richardson et al., 1990).

The type 2 astrocyte develops from a bipotential progenitor, known as the O2A cell , found in the rat optic nerve around birth (Raff et al., 1983). This cell also gives rise to oligodendrocytes *in vivo* and *in vitro*. In the optic nerve, the O2A progenitors proliferate, until at the time of birth some cells stop dividing and differentiate into oligodendrocytes. Others continue to proliferate and asynchronously stop dividing over the next few weeks to generate oligodendrocytes (Raff, 1989; Richardson et al., 1990). When cells from the optic nerve are placed in culture in low (>0.5%) serum concentrations, the cells all cease division and differentiate into oligodendrocytes. Addition of astrocyte conditioned medium restores the ability of the progenitors to proliferate and prevents premature differentiation into oligodendrocytes (Raff et al., 1985). It has been shown that platelet-derived growth factor-AA (PDGF-AA) can restore the timing of progenitor differentiation *in vitro* (Raff et al., 1988). PDGF-AA and insulin-like growth factor-1 (IGF-1) are survival factors for the progenitors in culture (Barres et al., 1992). Addition of NT-3 and PDGF-AA promotes proliferation of the progenitors, with differentiation into oligodendrocytes occuring synchronously within a clone of cells, suggesting that the number of cell divisions is strictly limited (Barres et al., 1994a). Addition of CNTF to the growth factor combination prevents differentiation of the progenitors. These cells can also be made to proliferate indefinately without oligodendrocyte differentiation in the presence of PDGF and bFGF (Bögler et al., 1990). Oligodendrocytes have different growth factor requirements to progenitors: after differentiation, PDGF-AA is no longer able to prevent the death of the differentiated cells. However, IGF-1, a neurotrophin such as NT-3 or a neurokine, such as CNTF support short-term survival of oligodendrocytes.

A combination of at least two factors from the different classes is required for long term survival (Barres et al., 1993).

The timing of oligodendrocyte differentiation is believed to depend on an intrinsic clock mechanism that counts the number of divisions made by the progeny of a single cell (Raff, 1989; Richardson et al., 1990) After a preset number of divisions, approximately 8, the clone of cells produced will become unresponsive to mitogens and undergo more or less synchronous differentiation into oligodendrocytes (Raff et al., 1985; Barres et al., 1994a). The asynchronous production of oligodendrocytes within the developing optic nerve is thought to reflect the timing of generation of the initial progenitor cell (Barres et al., 1994b). Recently, the processes controlling the intrinsic clock have been partly elucidated. There appears to be two parts to the timing mechanism: a counting component that involves proliferation driven by mitogens, and an effector mechanism that depends on hydrophobic signalling molecules including thyroid hormone, retinoic acid and glucocorticoids (Barres et al., 1994b). It has been suggested that the susceptibility of progenitors to differentiate in response to the effector signals increases with every round of cell division. The intermediary in this mechanism may be the transcription factor AP-1 which helps mediate the proliferative response to mitogens. The activity of AP-1 can be inhibited by the receptors for the above hormones. It is postulated that with every round of cell division the levels of AP-1 are decreased and at a particular level of the transcription factor the inhibitory effects of the hydrophobic signal receptors are sufficient to block its activity and to cause withdrawal of the cell from the cell cycle. It is proposed that this process is sufficient to cause oligodendrocyte differentiation (Barres et al., 1994b).

Growth factors and the nervous system

Our understanding of the role of growth factors in the development and homeostasis of the nervous system has increased enormously in the decades since the first effects of NGF on neuronal survival were reported by Levi-Montalcini and Hamburger (reviewed in Levi-Montalcini, 1987). Growth factors are required by cells of both the developing and mature nervous system for survival and proliferation (reviewed in Lemke, 1990; Barres et al., 1992, 1993, 1994a; Snider, 1994) and now there is increasing evidence that growth factors control major developmental decisions. Studies on the differentiation of neural crest cells (described in the following section)

have revealed that many growth factors can direct crest cell differentiation towards a particular fate. Some of the growth factors known to have a role in the PNS, with particular reference to Schwann cells, will be described here. The growth factors of the fibroblast growth factor (FGF) family, the insulin growth factor (IGF) family and the Neu differentiation factor (NDF) family are described in Chapters 5 and 6.

PGDF-AA and -BB

PDGF is found in three isoforms that are disulphide-bonded dimers of A and B chains: AA, BB and AB (reviewed in Heldin and Westermark, 1990). The receptors for PDGF have tyrosine kinase activity and are comprised of α and β chains that dimerise on binding the PDGF ligand; homodimers of αα or ββ and the heterodimer of αβ are formed. The α receptors can bind both the A and B chain of PDGF but the β receptor appears to bind the B chain alone (Heldin et al., 1988). Dimerization of the receptors leads to autophosphorylation, with subsequent phosphorylation of cytoplasmic substrates (Eriksson et al., 1992; Schlessinger and Ullrich, 1992).

PDGF is a potent mitogen and chemotactic agent for cells derived from the mesenchyme (Heldin and Westermark, 1990). It has also been shown to have a major role in CNS glial development, acting a mitogen for oligodendrocyte progenitors and affecting the timing of oligodendrocyte differentiation as mentioned above (Raff et al., 1988; Richardson et al., 1988; Pringle et al., 1989; Barres et al., 1993). In the PNS, PDGF-BB is a mitogen in the presence of elevated cyclic AMP for rat Schwann cells (Davis and Stroobant, 1990; Weinmaster and Lemke, 1990) and can act in an autocrine manner (Eccleston et al., 1990).

In the developing embryo, all three isoforms of PDGF are found within the developing CNS and PNS (Yeh et al., 1991; Hutchins and Jefferson, 1992; Reddy and Pleasure, 1992). Mouse motor neurones are immunoreactive for all three PDGF isoforms at E12.5 and immunoreactivity is detected in DRG neurones and peripheral nerve one day later (Hutchins and Jefferson, 1992). There is expression of PDGF in neonatal and adult rat Schwann cells, with levels peaking shortly after birth (Eccleston et al., 1993).

E13.5 mouse nerve fibres express PDGF β receptor protein (Hutchins and Jefferson, 1992) and there is immunohistochemical evidence that rat Schwann cells express the

both the α and β receptor at P1 (Eccleston et al., 1993). The detection of α receptor protein is in contrast with pharmacological evidence that suggests that α receptors are absent or non-functional on Schwann cells, since PDGF-AA, in contrast to PDGF-BB, has no known effect on Schwann cells (Davis and Stroobant, 1990).

Neurotrophic factors - NGF, BDNF, NT-3, NT-4/5

In addition to the effects of neurotrophins on the neural crest (see below), these factors have been shown to support the survival of various neuronal populations in peripheral sensory and sympathetic ganglia as well as motor neurones (reviewed in Levi-Montalcini, 1987; Thoenen, 1991; Henderson et al., 1993; Klein, 1994; Snider, 1994; Snider and Wright, 1996).

The neurotrophins interact with three high affinity tyrosine kinase receptors, trkA, trkB and trkC, and the low affinity p75LNGFr. The neurotrophins bind to the receptors in a highly specific manner: the trkA receptor binds primarily NGF but will also bind NT-3; the primary ligands for trkB are BDNF and NT-4, with NT-3 also able to bind to this receptor; trkC appears to be specific for NT-3 (reviewed in Snider, 1994). Both trkB and trkC have truncated isoforms lacking the tyrosine kinase domain, at least two truncated forms exist for trkB and four for trkC (Snider, 1994). The p75LNGF receptor can bind all the neurotrophins with low affinity but the exact function of this receptor is not known (Meakin and Shooter, 1992; Curtis et al., 1995).

The neurotrophins assessed for Schwann cell precursor survival in the present work (Chapter 5) are all found within the peripheral nervous system. BDNF and NT-3 are expressed by neurones of peripheral ganglia (Ernfors and Persson, 1991; Schecterson and Bothwell, 1992) and Schwann cells can produce NGF and BDNF after nerve lesion, as described above (reviewed by Scherer and Salzer, 1996). Both trkB and trkC are found on migrating crest cells that will form the PNS (Klein et al., 1990; Tessarollo et al., 1993). Schwann cells in normal nerves express truncated forms of trkB and trkC, as well as full length trkC from E16 (the earliest time point studied) but the full-length trkC is down-regulated in the adult (Scherer and Salzer, 1996; Offenhäuser et al., 1995). It is not known what function the truncated receptors perform in cells but it has been postulated that they may negatively modulate the effects of neurotrophins by preventing the interaction with full length receptors

(Offenhäuser et al., 1995). As described earlier, p75LNGFr is present on cells of the Schwann cell lineage until they differentiate into myelin-forming Schwann cells, with expression being maintained on non-myelin-forming Schwann cells.

Neurokines - CNTF, LIF

Ciliary neurotrophic factor (CNTF) and leukaemia inhibitory factor (LIF) are related cytokines that exhibit trophic effects on cells of the nervous system (reviewed in Rao and Landis, 1993). Both factors promote the survival of sensory and motor neurones, with CNTF also promoting sympathetic neuronal survival (reviewed in Adler, 1993; Sendtner et al., 1990; Murphy et al., 1991; Thoenen, 1991; Rao and Landis, 1993; Mitsumoto et al., 1994). CNTF, LIF, and the related cytokine IL-6, promote the long term survival of oligodendrocytes *in vitro* (Barres et al., 1993, 1994) and CNTF can prevent oligodendrocyte death *in vivo* (Barres et al., 1993).

The receptors for these neurokines are integral membrane proteins and share the common subunit gp130; the LIF receptor comprises gp130 and the LIFβ receptor subunit; the CNTF receptor has the additional CNTFα receptor complexed with both gp130 and LIFβ receptor (Scherer and Salzer, 1996).

Both CNTF and LIF are expressed by neonatal Schwann cells, CNTF expression is low during the first postnatal week and increases thereafter, with high levels of expression in myelinating Schwann cells (Rende al., 1992; Curtis et al., 1993, 1994).

SCF/ Steel factor

Stem cell factor (SCF) is also known as Steel factor, *kit* ligand or mast cell growth factor. Its receptor, c-kit, is a protein tyrosine kinase with homology to the PDGF receptor (Qui et al., 1988). SCF and c-kit were discovered to have a role in melanogenesis from two mouse mutants, *Steel* (*Sl*)and *Dominant White Spotting* (*W*) (reviewed by Williams et al., 1992). These mutations, lacking SCF and c-kit respectively, are both lethal in homozygous mice, but heterozygous mice survive and display a white spotted coat colour pattern. The pattern of SCF and c-kit expression in the normal mouse shows an extensive distribution of transcripts, cells other that those derived from the neural crest also express these proteins (Steel et al., 1992). Steel factor is normally expressed as two splice-variants, both are membrane bound but the larger variant contains an extracellular cleavage site. In the *Steel-dickie* (Sl^d) mouse

mutant, a deletion of the transmembrane and cytoplasmic regions of the Sl gene produce a secreted form of the factor. By analysing neural crest migration in Sl^d mutants, Wehrle-Haller and Weston (1995) have shown that the secreted form of SCF is a survival factor for melanocyte precursors in the initial staging area (between the neural tube, somite and overlying ectoderm) and during their dispersal on the dorsolateral pathway (see below). Survival of the cells once they are in the dermis, however, appears to require the presence of the membrane bound form of SCF, but this factor does not appear to be required for differentiation into melanocytes (Murphy et al., 1992; Steel et al., 1992).

SCF is found within cells of the mouse embryonic DRG, but there is no apparent expression of SCF in the adult DRG (Hirata et al., 1995). The c-kit receptor is expressed on melanoblasts and on cells of embryonic and postnatal mouse DRG (reviewed by Morrison-Graham and Takahashi, 1993; Hirata et al., 1995).

Transforming growth factor βs

TGFβ1 and TGFβ2 are members of the TGFβ superfamily of growth factors (reviewed by Kingsley, 1994). They are synthesised as precursor proteins each containing an amino-terminal signal sequence, a pro-domain and, at the carboxy-terminal, the bioactive domain. The precursors form dimers that are then cleaved by proteolysis producing mature TGFβ. The cleaved TGFβ may remain non-covalently linked to the pro-domain, forming a latent complex that can be activated by proteolysis or low pH (Kingsley, 1994; Massagué et al., 1994).

The TGFs bind to and signal via two main receptors known as type I and type II TGFβ receptors, (TGFβR) both are serine/threonine kinases. Although TGFβ can bind to the type II TGFβR alone, both receptors are required for signalling (Massagué et al., 1994; Kingsley, 1994). A third receptor has been described, the type III receptor, which is the proteoglycan betaglycan. This is believed to regulate the binding of TGFβ isoforms to the type II receptor (Kingsley, 1994).

Schwann cells express all three TGFβ receptors (Einheber et al., 1995). The presence of TGFβ receptors on cells of the neural crest can be inferred from changes in neural crest phenotype after TGFβ addition (Stocker et al., 1991; Delannet and Duband, 1992; Rogers et al., 1992; Leblanc et al., 1995), for example: a novel member of the

TGFβ family, *dorsalin-1*, promotes the differentiation and migration of neural crest cells from the neural tube (Basler et al., 1993).

Both Schwann cell precursors and Schwann cells express TGFβs 1-3 in latent form (Ridley et al., 1989; Einheber et al., 1995; Scherer and Salzer, 1996; Stewart et al., 1995). Peripheral neurones in embryonic mice are immunoreactive for TGFβs 2 and 3 (Flanders et al., 1991), while neonatal rat DRG neurones secrete active TGFβ in short-term cultures (Stewart et al., 1995).

TGFβs have been shown to have many effects on Schwann cell phenotype and behaviour including down-regulation of myelin protein expression (Morgan et al., 1994; Guérnard et al., 1995), upregulation of the cell adhesion molecules N-CAM and L1, and stimulation of proliferation in the presence of serum or FGF (Eccleston et al., 1989; Ridley et al., 1989; Schubert, 1992).

The neural crest

As mentioned earlier, the cells of the neural crest give rise to most of the neurones and glia in the PNS. In addition, the neural crest generates many other cell types, including melanocytes, neurosecretory cells of the adrenal medulla, carotid body and parathyroid glands, as well as skeletal and connective tissue of the skull (Le Douarin and Smith, 1988; Anderson, 1989).

Neural crest cells are a transient population that form at the edges of the neural plate as a result of inductive events between the neural plate and the presumptive ectoderm (reviewed in Selleck et al., 1993; Bronner-Fraser, 1995; Selleck and Bronner-Fraser, 1995). The neural plate folds to produce the neural tube during neurulation and, at the time of closure, the neural crest cells separate from the neural folds to migrate along defined pathways within the developing embryo. The migration pathways of these cells can be extensive and depend on the rostro-caudal position within the embryo. Once migration has ceased, the cells differentiate into appropriate cell types depending on the location (Le Douarin and Smith, 1988).

How the cells of the neural crest generate such a diversity of cell types and at precise locations within the developing embryo is not yet fully understood. The timing and pathways of migration play a major role in determining the progeny of the neural crest (Bronner-Fraser, 1993). Studies of neural crest migration *in vivo* have revealed some

of the environmental cues that control the routes taken by the neural crest cells (reviewed in Erickson and Perris, 1993). *In vivo* studies of labelled single cells have revealed that the progeny of a single neural crest cell can generate cells that contribute to several lineages within the developing embryo, implying that the original cells are multipotent, and the same conclusion has been drawn from experiments on clonal cultures of neural crest cells (see below) (reviewed in Bronner-Fraser, 1995; Bronner-Fraser and Fraser, 1988, 1989; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). Recent work on the control of developmental decisions in other cell systems and *in vitro* studies of neural crest have provided insights into possible mechanisms for directing neural crest cell differentiation towards a particular cell lineage.

Pathways of migration

Migration of crest cells *in vivo* has been studied using a variety of methods. Initial studies used transplantation of donor tissue labelled with tritiated thymidine (Weston, 1963), while later transplantation experiments used chick / quail chimeras, exploiting the visible species differences of the cell nucleoli to distinguish the migrating crest cells (Le Douarin and Teillet, 1974; Le Douarin and Smith, 1988). Migration of neural crest cells in the chick and rat has also been examined using the antibodies HNK-1 and NC-1 that recognise a glycosylated epitope present on a variety of cell types (Vincent, et al., 1983; Tucker, et al., 1984; Loring and Erickson, 1987; Erickson et al., 1989). It is expressed on the surface of most chick neural crest cells and some of their derivatives (Vincent, et al., 1983; Tucker, et al., 1984) However, the epitope is expressed only transiently on some cells, particularly those that go on to generate melanocytes (Vincent, et al., 1983; Tucker, et al., 1984; Bronner-Fraser, 1986; Loring and Erickson, 1987; Dupin, et al., 1990; Heath, et al., 1992). It is expressed only on a subset of rat neural crest cells (Erickson et al., 1989; Bannerman and Pleasure, 1993). More recently, the vital dye DiI has been used to label premigratory crest cells in both chick and mouse (Serbedzija et al., 1989, 1990, 1991, 1992). This lipophilic dye is incorporated into the cell membranes and labels all cells that are in contact with the lumen of the neural tube. Examining embryos at successive stages after injection has revealed the migration paths of neural crest cells and by altering the time of injection, the chronological order of crest cell contribution to various derivatives has been determined (reviewed in Bronner-Fraser, 1995). All these methods have given similar

results, with comparable patterns of migration observed in chick, mouse and rat (Bronner-Fraser, 1986; Erickson et al., 1989; Serbedzija et al., 1989, 1990).

Migration proceeds in a rostro-caudal manner; the first neural crest cells migrate from the neural tube at the level of the most recently formed somite (reviewed in Erickson and Perris, 1993). In the trunk region, the neural crest cells follow two main paths of migration: ventrally, through the rostral portion of the somites with some cells migrating in the intersomitic space, and dorsolaterally, between the ectoderm and the dermomyotome (reviewed in Bronner-Fraser, 1993; Bronner-Fraser, 1986; Erickson et al., 1989; Serbedzija et al., 1989, 1990).

In the ventral pathway, the caudal half of the somite is non-permissive for neural crest migration and also for motor axon outgrowth, resulting in the segmental patterning observed in the developing PNS (reviewed in Erickson and Perris, 1993; Keynes and Stern, 1984; Rickmann et al., 1985; Bronner-Fraser, 1986; Loring and Erickson, 1987; Bronner-Fraser and Stern, 1991). The restriction of crest cell migration to the rostral half of the somite results in a temporal delay in migration of cells facing the caudal half of the somite. These cells wait at the dorsal surface of the somite before moving either in an anterior direction to the migration permissive, rostral half of the somite, or caudally to the rostral half of the adjacent somite (reviewed in Le Douarin and Dupin, 1993). Ventral migration of the neural crest is affected by the notocord which blocks the passage of the cells through the sclerotome in the vicinity of the notocord, possibly as a result of an inhibitory factor (reviewed in Bronner-Fraser, 1995).

The neural crest forms derivatives in a ventral-to-dorsal manner within the embryo (Serbedzija et al., 1989; 1990; Bronner-Fraser, 1993). In the mouse, two patterns of migration are evident in cells following the ventral pathway with early migrating cells taking a pathway along the ventrolateral portion of the sclerotome to populate the sympathetic ganglia, adrenal medulla and the aortic plexus, while later migrating cells take a ventromedial route between the neural tube and the rostral sclerotome, moving into the sclerotome to form the DRG (Serbedzija et al., 1989; 1990). In the chick, the ventrally migrating neural crest cells appear to follow a single pathway to populate both sympthoadrenal derivatives and DRG (Rickmann et al., 1985; Bronner-Fraser, 1986).

Cells migrating on the dorsolateral pathway appear to give rise exclusively to melanocytes (Erickson et al., 1992; Erickson and Perris, 1993; Erickson and Goins, 1995). Migration of these cells is observed later than that of cells following the ventral route; the cells delay entering the pathway by up to one day after cells from the same axial level have entered the ventral pathway. Conditions generated by the dermomyotome appear to be responsible for the failure of the crest cells to migrate earlier on this pathway (Erickson et al., 1992).

The pathways followed by the neural crest during migration provide substrates consisting of ECM molecules such as fibronectin, laminin and collagen, that are favourable to crest cell motility (Erickson and Perris, 1993). Conversely, areas such as the caudal half-somite and around the notocord are not permissive for neural crest migration, restricting the movement of these cells (Keynes and Stern, 1984; Rickmann et al., 1985; Norris et al., 1989). Thus, neural crest cells are subject to external influences on the pathways of migration they can take. In addition, the timing of migration can be controlled by cells surrounding the neural crest. Temporally expressed prohibitory signals appear to control the migration of cells following the dorsolateral pathway, an event that is likely to bias these cells towards a melanocyte fate (described below). Recent work has suggested that cells cannot migrate on the dorsolateral pathway unless specified as melanocytes (Erickson and Goins, 1995), however, studies *in vitro* have shown that the ability to migrate on the dorsolateral pathway does not preclude cells from adopting a non-melanocytic fate (see below) (Richardson and Sieber-Blum, 1993). It is probable that the restriction of neural crest cell migration plays a major role in controlling the final derivatives produced by crest cells, either due to intrinsically timed developmental events, or by exposure to extrinsic factors such as growth factors, which may bias the type of cells formed from a multipotent progenitor.

The choice of cell fate

The cell types generated from the neural crest depend on the axial level of origin of the crest cells. Tranplantation studies using chick-quail chimeras have shown that mesectodermal derivatives are produced exclusively from crest migrating from the cranial region of the embryo and not from crest cells arising in the trunk region (reviewed in Le Douarin and Smith, 1988). Similarly, enteric neurones and glia are

generated from the most rostral trunk neural crest (with a subpopulation from sacral crest) but most of the trunk crest cells do not contribute to the generation of the enteric nervous system (Le Douarin and Smith, 1988; Serbedzija et al., 1991; Gershon et al., 1993). It is possible that these cells are predetermined populations that migrate to their final destinations instructed by specific cues in the environment. However, the chick-quail chimeric work by Le Douarin and collegues (reviewed in Le Douarin and Smith, 1988; Le Douarin and Dupin, 1993; Le Douarin et al., 1993) has shown that neural crest cells transplanted to a new position on the rostro-caudal axis will give rise to many crest progeny appropriate to this new axial position, suggesting that the cells are not predetermined but are influenced by environmental factors in their choice of cell fate. However, although cranial neural crest transplanted to the trunk region of the embryo can generate the full range of trunk crest derivatives, converse experiments show that trunk crest is unable to generate mesectodermal derivatives when transplanted to the cranial region (Le Douarin and Smith, 1988; Le Douarin et al., 1993) suggesting a restriction in the potential fates of the trunk crest.

The generation of diverse cell types from an homogenous population of cells occurs in many different cell systems during development. All cells in a population that have the potential to make a cell fate decision with equal probability can be thought of as an equivalence group (reviewed in Greenwald and Rubin, 1992). The choice of cell fate may be influenced by signals generated outside this group, an event known as induction. Alternatively, a stochastic event in one of the cells, such as a minor change in cell surface signalling molecules, may generate non-equivalence within the group. This alteration of equivalence can produce lateral specification, with the initially non-equivalent cell preventing the other cells from differentiating along the same pathway and making them adopt an alternative fate (Greenwald and Rubin, 1992).

In the haemopoietic system, most cells involved in the immune response are generated from stem cells in the bone marrow (reviewed in Lydyard and Grossi, 1989). The multipotent stem cells give rise to cells that are committed to one of four cell lineages. These cells in turn produce more restricted progenitors in the lineages, eventually producing terminally differentiated cells. The stem cells can be cultured *in vitro* in the presence of polypeptide growth factors such as the interleukins (IL-) 1 to 11 and the haemopoietic colony stimulating factors (CSFs); the cell types generated can be

influenced by the growth factors present (Fairbairn et al., 1993). These growth factors are known to promote survival and proliferation of the stem cells progeny but it was previously unknown whether the growth factors induced differentiation of a particular cell type. By suppressing death in the stem cell population using transfection with the anti-apoptotic gene *bcl-2* (described in Chapter 4), Fairbairn et al. (1993) showed that stem cells *in vitro* generated differentiation of all the normal progeny in the absence of exogenous growth factors. This suggests that these cells generate different cell types by stochastic events, with growth factors biasing the proportions of each subpopulation by subsequent survival and proliferation effects. How cell fate decisions are made in the developing PNS is as yet unclear, but studies with neural crest cells *in vitro* have provided insights into factors controlling differentiation of these cells.

In vitro studies on trunk neural crest cells

As discussed above, labelling of single cells *in vivo*, either by transfection with retrovirus or by DiI injection, has revealed that at least some cells of the neural crest are multipotent (Bronner-Fraser and Fraser, 1988, 1989; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991; Serbedzija et al., 1994). These studies also suggest that cells with a more restricted potential can be found within the neural crest, and that with time the potential fates of the crest cells becomes increasingly restricted, confirming earlier work with chick/quail chimeras using back transplantation (Le Douarin and Smith, 1988). Although such work can suggest possible sublineages and timing of committment within this cell population, the factors controlling the direction of neural crest differentiation cannot be resolved using these methods. Using *in vitro* culture conditions, however, the effects of individual growth factors on crest cell development can be studied.

Several types of study have been performed using cultured neural crest cells (reviewed in Stemple and Anderson, 1993). Explants of neural tubes producing mass cultures of neural crest have been used to analyse the effects of substrates and growth factors on whole populations. An alternative method has been to culture neural crest cells at clonal density, thereby controlling more closely the factors affecting crest cell development. This method allows the fate of single cells to be followed, and has produced evidence for progenitors with variously restricted potentials.

Using a clonal culture method, rat neural crest cells capable of undergoing self-renewal have been detected (Stemple and Anderson, 1992). It has been argued that these cells act as stem cells, capable of producing multipotent progeny even after undergoing several rounds of division. Within these cultures, however, cells with a more restricted potential are also found, confirming previous work in chick, quail and mouse (Cohen and Konigsberg, 1975; Sieber-Blum and Cohen, 1980; Barrofio et al., 1988; Dupin et al., 1990; Ito and Sieber-Blum, 1991; Ito et al., 1993). In all these studies, some clones produced cells of many different lineages, with melanocytes, Schwann cells, serotonergic (5-HT$^+$) and substance P$^+$ neurones differentiating from a single progenitor (Dupin et al., Ito et al., 1993). In contrast, some clones produced pigmented cells alone, suggesting that segregation of the melanocyte lineage is an early event in crest cell development. However, differences in the proportion of colonies giving rise to these pigmented clones occur in mouse (8%; Ito et al., 1993) and quail (40-60%; Sieber-Blum and Cohen, 1980) suggesting that timing or control of these lineages differs between species.

Clones with apparently tri- and bipotential precursors have been detected in quail and mouse crest cell cultures. The existence of bi- or tripotent progenitors has been inferred from the observation that a certain proportion of clones regularly give rise to just two or three cell types. For example, in quail cultures, clones are generated that contain only Schwann cells (SMP$^+$) and satellite cells (SMP$^-$); also a bipotential progenitor for Schwann cells and melanocytes has been suggested (Ciment, 1990). In mouse cultures, clones generating Schwann cells and melanocytes have also been found, as well as clones that give rise to only serotonergic and sensory neurones (Ito et al., 1993). A proposed tripotential progenitor in quail produces these cells plus neurones (Dupin et al., 1990). These studies suggest that, as well as multipotential progenitors, some cells of the neural crest exhibit more restricted potentials.

In two studies where cranial crest was cloned, mixed and single cell type clones of connective tissue and muscle derivatives developed, cell types that were not seen in cultures of trunk neural crest (Dupin et al., 1990; Ito and Sieber-Blum, 1991; Ito et al., 1993). These results again confirm data from transplantation studies (Le Douarin and Smith, 1988) suggesting that cells derived from the truncal level are already restricted in potential compared to cells of cranial crest.

Clonal analysis has also been performed on tissues populated by neural crest cells after crest cell migration has ceased. In cultures of quail enteric neural crest taken from the gizzard and bowel, clones can be generated that express tyrosine hydroxylase, a marker for entry into the sympathoadrenal lineage (reviewed in Anderson, 1993b; Sextier-Sainte-Claire Deville et al., 1994). Similarly in studies using early DRG and sympathetic ganglia, multipotential clones can be generated that give rise to melanocytes, sensory and autonomic neurones (Duff et al., 1991). Melanogenic potential of the clones is lost with increasing age of the donor embryo, again suggesting that commitment to the melanocyte lineage is an early event. Clones containing neuronal precursors of either sensory or sympathoadrenal lineages are only found in the cultures of the appropriate ganglia. Melanocytes appear to be among the first cells to become restricted *in vitro*, but culture of crest-derived cells from quail skin shows that some of these cells are also multipotential. Sensory neurones, sympathoadrenal cells and other non-pigmented cells (possibly glia) are produced by 20% of clones (Richardson and Sieber-Blum, 1993). In all the above cultures, production of these multipotential clones decreases with increased age of the embryo used to isolate the crest derivatives. These studies confirm the data produced by back transplantation studies in the chick, that the developmental potentials of cells derived from the neural crest are plastic and can be altered by changes in the environment. This plasticity is lost with increasing age as cells appear to become terminally committed to a particular cell lineage.

Effects of growth factors on neural crest development

In the cultures described above, the culture media contain unknown growth factors that are added in the form of serum and chick embryo extract, or factors secreted by fibroblast feeder layers. While most of the experiments described below are not performed entirely in defined media, addition of known growth factors to the cultures can be seen to have profound effects on the development of subsets of neural crest derivatives.

FGFs A role for FGFs in neural crest survival originally came from *in vivo* studies where a silastic membrane was inserted between the neural tube and the developing DRG anlage (Kalcheim and Le Douarin, 1986). This lead to death of the cells distal to the membrane, but coating the membrane in laminin and bFGF restored the ability of

these cells to survive. In culture, bFGF has been shown to promote the survival of a non-neuronal subset of neural crest cells, but proliferation is unchanged in the presence of the growth factor (Kalcheim, 1989; Brill et al., 1992; Bannerman and Pleasure, 1993). Another study reports that the use of aFGF or bFGF in the presence of serum on mouse neural crest cultures stimulates proliferation of these cells (Murphy et al., 1991).

bFGF is reported to stimulate neuronal differentiation from avian neural crest cultures but only in a cell-mediated manner (Brill et al., 1992). Transfection of the baby hamster kidney cell line BHK-21 with human bFGF cDNA increases the number of neurones differentiating from co-cultured neural crest. Parental cells fail to promote neuronal differentiation in the absence of bFGF but addition of exogenous growth factor to these cells also increases the numbers of neurones in the cultures. In contrast, in rat neural crest cultures addition of aFGF and bFGF are reported to delay the maturation of neurones (Bannerman and Pleasure, 1993; Bannerman and Guritzky, 1994).

bFGF has also been implicated in the development of melanocytes. Hirobe (1992) reported that bFGF in the presence of cAMP analogues increases the proliferation of mouse epidermal melanoblasts and prevents the differentiation of melanocytes. Addition of bFGF to embryonic DRG or peripheral nerve cultures stimulates the production of melanocytes (Stocker et al., 1991). The presence of the protein kinase C-activating phorbol, 12-O-tetradecanoyl phorbol-13-acetate (TPA) enhances the number of melanocytes produced, but the addition of TGFβ-1 reduces the number of pigmented cells developing. These results suggest that growth factor interplay may control the type of derivative formed in differentiating neural crest.

The FGFs have been implicated in the development of the sympathoadrenal lineage. Both sympathetic neurones and adrenal medulla chromaffin cells are derived from a bipotential progenitor (reviewed in Anderson, 1993a, 1993b). The differentiation of these cells is dependent on the environment, with the presence of glucocorticoids preventing neuronal differentiation while promoting the differentiation of chromaffin cells and subsequently acting as a survival factor for these cells (Doupe et al., 1985; Anderson, 1993a, 1993b). These cells can alter their phenotype even if isolated from the adult; in the presence of low glucocorticoid levels and NGF, these cells will

differentiate into sympathetic neurones (Doupe et al., 1985). The pheochromocytoma cell line PC12 resembles chromaffin cells in its response to growth factors and glucocorticoids and is often used to study sympathetic neuronal differentiation. Both bFGF and aFGF have been shown to mimic NGF effects on adrenal chromaffin cells and PC12 cells, by stimulating proliferation of these cells and inducing stable neurite outgrowth and neuronal differentiation (Rydel and Greene, 1987; Stemple et al., 1988; Claude et al., 1988). However, bFGF does not promote long-term survival of the differentiated neurones. After differentiation, the sympathetic neurones become dependent on NGF for their survival (Stemple et al., 1988).

These studies indicate that FGFs have a range of effects on the developing neural crest and survival, proliferation or differentiation in response to these factors depends on other signals from the environment.

Neurokines The neurokines LIF and CNTF have been implicated in neural crest cell differentiation along neuronal pathways. Mouse crest cells cultured in bFGF then switched to medium containing LIF or CNTF results in the differentiation of sensory neurones from these cultures (Murphy et al., 1991, 1994). Proliferation of chick sympathetic neuroblasts is inhibited by CNTF, which promotes the survival and differentiation of neurones from these cells (Ernsberger et al., 1989). In addition, CNTF appears to enhance production of neurones expressing vasointestinal peptide (VIP), normally only expressed by a subpopulation of sympathetic neurones (Ernsberger et al., 1989). It has been known for sometime that environmental factors can cause a switch in neuronal neurotransmitter, from a noradrenergic to a cholinergic phenotype (reviewed in Patterson, 1990; Doupe et al., 1985; Patterson and Chun, 1977). LIF and CNTF have also been reported to induce this switch *in vitro* in older sympathetic neurones cultured from neonatal rats (Yamamori et al., 1989; Ludlam et al., 1994) suggesting that these neurokines can determine the neurotransmitters expressed by neurones, and probably influence the neurones developing from the neural crest (reviewed in Rao and Landis, 1993).

Neurotrophins The neurotrophins NGF, BDNF and NT-3, have been implicated in several aspects of neural crest development (Sieber-Blum, 1991; Wright et al., 1992; Sieber-Blum et al., 1993). BDNF and NT-3 have been shown to promote the survival of neural crest cells. BDNF coating of silastic membranes implanted between the

neural tube and the DRG anlage, as described for FGF, prevents the death of developing DRGs suggesting a trophic role for BDNF (Kalcheim et al, 1987). NT-3 has also been shown to act as a survival factor for early neuroblasts differentiating from neural crest (DiCicco-Bloom et al., 1993). As described above, NGF can induce sympathetic neuronal differentiation and survival of sympathoadrenal progenitors (Anderson, 1993a, 1993b). In clonal cultures of quail trunk neural crest, addition of BDNF but not NGF promotes differentiation of sensory neurones (Sieber-Blum, 1991; Sieber-Blum et al., 1993). However, BDNF does not promote the proliferation of the cells in the clone, indicating that it acts to direct cell differentiation towards a sensory neurone fate (Sieber-Blum, 1991; Sieber-Blum et al., 1993).

NT-3 has been shown to promote the survival of sensory and sympathetic neurones in DRG and sympathetic ganglia (reviewed in Snider, 1994; Henderson, 1996). Culture of neural crest cells with NT-3 causes an increase in proliferation of these cells (Kalcheim et al., 1992). This factor promotes the survival of proliferating sensory neurone precursors (ElShamy and Ernfors, 1996); in this study, cultured DRG sensory neurone precursors from embryonic mice mutant for NT-3 show enhanced survival after the addition of exogenous NT-3. Both NT-3 and BDNF have been reported to increase the rate of neuronal maturation in cells from embryonic chick DRG (Wright et al., 1992).

Glial growth factor NT-3 has been shown to be produced by non-neuronal cells of sympathetic ganglia (Verdi et al., 1996) and it has been suggested that NT-3 and glial growth factor (GGF; Marchionni et al., 1993; described in Chapter 6), another growth factor implicated in neural crest differentiation, are both involved in cell fate determination within developing ganglia (Verdi et al., 1996). GGF, a member of the Neu differentiation factor family (also called neuregulins and heregulins), has been shown to bias neural crest differentiation towards a glial fate, suppressing neuronal differentiation while allowing glial differentiation to proceed (Shah et al., 1994; Mirsky and Jessen, 1996). In cultures of non-neuronal cells from sympathetic ganglia, NT-3 mRNA expression is upregulated by the addition of GGF-2, CNTF and at lower levels by PDGF. These cells produce a factor, identified as NT-3 by use of blocking antibodies, that can promote the survival of a subpopulation of sympathetic ganglia neuroblasts. In addition, NT-3 produced by the non-neuronal cells down-regulates the

expression of trkC and upregulates expression of trkA on the neuroblasts, conferring the ability to respond to NGF on these cells (Verdi and Anderson, 1994; Verdi et al., 1996). GGF mRNA can be detected in freshly isolated neuroblasts (Verdi et al., 1996) suggesting a possible mechanism for generating both neurones and glia within the developing ganglia: since cells differentiating early as neurones produce GGF they might then direct as yet uncommitted neighbouring cells towards a glial fate. These non-neuronal cells in turn produce NT-3 which can support the survival of the neuroblasts and affects the switch for trophic requirements in the differentiating neurones, from NT-3 in neuroblasts to NGF in neurones.

TGFβ superfamily The bone morphogenetic proteins (BMPs) are members of the TGFβ superfamily and are expressed at sites of autonomic neuronal differentiation (Shah et al., 1996). BMP-2 induces stem cell cultures of rat neural crest to differentiate into neurones of the sympathetic lineage. Some clones containing only neurones develop under these conditions, indicating that BMP-2 is likely to act directly on the cells, rather than via a non-neuronal intermediate. Some clones produce cells expressing smooth muscle antigens, and in cultures where TGFβ1 is used instead of BMP-2, virtually all cells differentiate into smooth muscle (Shah et al., 1996). Smooth muscle is not normally associated with derivatives of trunk neural crest and although showing that these cells have not lost the potential to differentiate along this pathway, may not represent actual cell fate decisions made *in vivo*.

Stem cell factor (SCF) As described above, the membrane-bound form of SCF has been shown to be a survival factor for melanocyte precursors *in vivo* (Steel et al., 1992) and *in vitro* (Murphy et al., 1992). Addition of soluble SCF acts as a survival factor for embryonic mouse sensory neurones *in vitro* (Hirata et al., 1995). Most recently SCF has been shown to be trophic for pluripotent quail neural crest cells *in vitro*. In addition, it enhances the number of sensory neurone precursors developing from these cultures, when present on its own, but in combination with NGF or NT-3, this effect is lost. However, in combination with a neurotrophin, SCF promotes the survival of melanogenic cells, suggesting multiple actions of this factor during neural crest cell differentiation (Langtimm-Sedlak et al., 1996).

In conclusion, the development of a particular cell type from the multipotent cells of the neural crest involves a series of complex events, from interaction with the ECM, to

cell-cell interactions and the effects of growth factors and hormones in the environment. The cell ultimately differentiating *in vivo* at the end of development is a sum of all these events. The work presented here is a study of a cell that is an intermediate on the path of development from the neural crest to the Schwann cell. The phenotype of this cell, its survival requirements and the factors involved its maturation to a Schwann cell phenotype will be described in the following chapters.

Table 1.1 Molecular phenotype of rat peripheral glia

A comparison of some antigens expressed by glial cells of the adult rat PNS *in vivo* and short-term cultured Schwann cells. (Data from Bannerman et al., 1988; Mirsky and Jessen, 1987, 1990; Gould et al., 1992; * R. Mirsky, personal communication).

Table 1.1 Molecular phenotype of rat peripheral glia

Antigen	Satellite cells	Enteric glia	Non-myelin-forming Schwann cells	Myelin-forming Schwann cells	Short-term cultured Schwann cells
p75LNGFr	+	–	+	–	+
S100	+	+	+	+	+
N-CAM	+	+	+	–	+
L1	+	+	+	±[a]	+
A5E3	+	+	+	–	+
Ran-2	–	+	+	–	+
Laminin	+	+	+	+	+
GAP-43	+	–	+	–	+
Vimentin	+	+	+	+	+
GFAP	+[b]	+	+	+	+
04	+	+	+	+	–
GalC	+[*]	–	+	+	–
P_0	–[c]	–[c]	–[c]	+	–[c]
MBP	–	–	–	+	–

[a] expressed mainly at nodes of Ranvier.

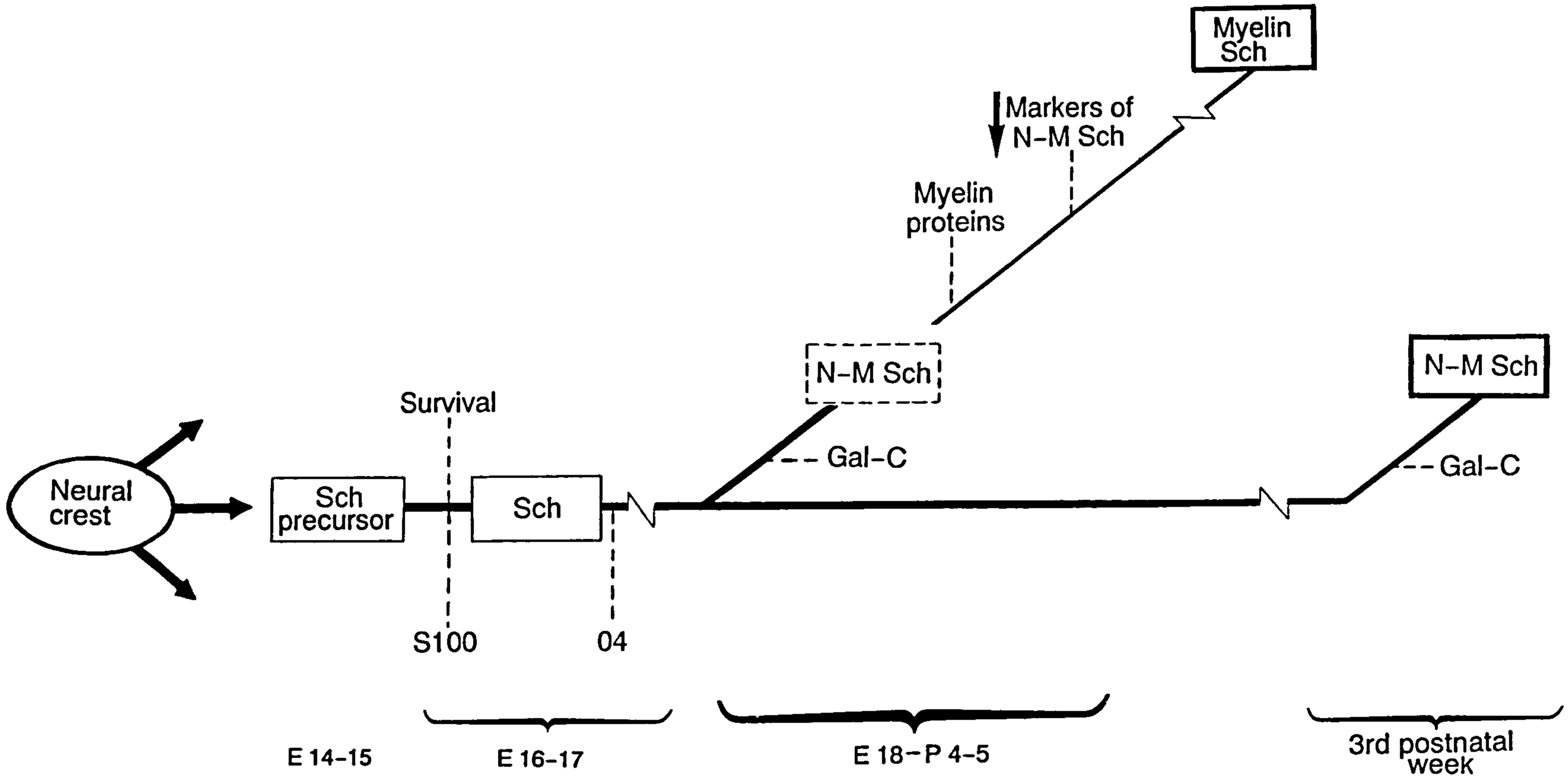
[b] expressed in 40-60% of rat sympathetic ganglia satellite cells but only present in a minority of DRG satellite cells.

[c] expressed at basal levels but not at high levels, basal levels in enteric glia and satellite cells not determined.

Figure 1.1 Schematic representation of rat Schwann cell development in the sciatic nerve.

The scheme describes the observed stages in development of rat Schwann cells from cells of the neural crest. Schwann cell precursors are found in the nerve at E14-15. Schwann cells are generated from Schwann cell precursors between E15-17, when S100 is first detected and the cells survive culture in defined conditions (Chapter 3). At around birth, the first cells adopting the myelin phenotype are seen, with expression of P_0 being observed. By the fifth post-natal day, the first cells to express myelin proteins have down-regulated molecules associated with the mature non-myelin-forming Schwann cells, such as GFAP, NCAM, A5E3 and p75LNGFr. Mature non-myelin-forming Schwann cells, expressing GalC are found in the third postnatal week. Differentiation of Schwann cells in the nerve is complete by the fifth to sixth postnatal week.

Sch, Schwann cell; N-M Sch in solid frame, non-myelin-forming Schwann cell; N-M Sch in stipple frame, cells with a similar molecular phenotype to the mature non-myelin-forming Schwann cell that will progress to myelination; M-Sch, myelin-forming Schwann cell; GalC, galactocerebroside. ↓ indicates molecules that are down-regulated during differentiation of Schwann cells along the myelin pathway. Myelin proteins are those normally expressed only by myelin-forming Schwann cells, such as the proteins P_0, MBP, and MAG. Reproduced by the kind permission of R. Mirsky and K. R. Jessen (Jessen and Mirsky, 1992).


Myelin Sch
Markers of N-M Sch
Myelin proteins
N-M Sch
Gal-C
N-M Sch
Gal-C
Survival
Neural crest
Sch precursor
Sch
S100
04
E 14-15
E 16-17
E 18-P 4-5
3rd postnatal week

CHAPTER 2

MATERIALS AND METHODS

Animals

Sprague Dawley rats were used throughout this work.

Pregnant rats were killed by asphyxiation in CO_2, and the embryos decapitated and kept on ice. Neonates were killed by decapitation.

Dating of the embryos was as described in Christie, 1965, taking the day of the vaginal plug to be embryo day (E) 0.

Defined Medium

The defined medium (DM) used in this study was a modification of the medium of Bottenstein and Sato (1979). Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (both Gibco Life Technologies, Paisley, Scotland) mixed 1:1 and supplemented with (final concentrations in parenthesis) bovine serum albumin (BSA, 0.03 mg/ml), transferrin (100µg/ml), glucose (7.9mg/ml), putrescine (16µg/ml), progesterone (60ng/ml), thyroxine (400ng/ml), triiodothyronine (10.1ng/ml), selenium (160ng/ml), dexamethasone (38ng/ml) (all the above were from Sigma Chemical Company, Poole, England), glutamine (2mM; ICN Biomedicals, Thame, England), penicillin (100 IU/ml) and streptomycin (100 IU/ml) (both from Gibco Life Technologies). Insulin or IGFs were used separately or together at concentrations appropriate for the experiment (detailed in the text).

Defined medium with components omitted (Chapter 5)

The hormones progesterone, triiodothyronine, thyroxine and dexamethasone were omitted from defined medium either singly or all together. Survival experiments in these media were performed by plating the cells in deficient medium containing 100ng/ml IGF-1 and, after 3hr, adding 280µl of the same medium containing 100ng/ml IGF-1 and 3ng/ml bFGF.

Preparation of coverslips and substratum coating

13mm diameter coverslips (BDH, Lutterworth, England) were sterilized by baking at 140°C for 4 hr. Coverslips were coated with poly-L-lysine (PLL; >300,000 MW, Sigma) by incubating with a solution of 1mg/ml PLL in deionised H_2O for 24 hr at room temperature (RT). The coverslips were then washed over 3 days with 8 changes

of sterile deionised water and air dried. The coverslips were stored dessicated for at least one month prior to use.

Wells of plastic 4-well multidishes (Nunc, Gibco Life Technologies, Scotland) were coated with 250µl of a lower concentration PLL solution, 20µg/ml, and incubated for 2 hr at RT. The solution was removed and the wells were air dried without washing, and kept for a week prior to use.

For laminin coating, coverslips or wells previously coated with PLL were used. Laminin (Gibco Life Technologies) was diluted in DMEM to a final concentration of 20µg/ml from a 1mg/ml stock solution and incubated on coverslips at RT for 1 hr. The volume of laminin used was either 10 or 20µl on coverslips and 50µl in wells.

Fibronectin (Gibco Life Technologies) was diluted from a 1mg/ml stock solution in DMEM to a final concentration of 25µg/ml and incubated on PLL coverslips for 1 hr at RT, as above.

Collagen, in the form of acid soluble rat tail collagen (produced by the method described in Bergsteinsdottir et al., 1993), was used at an approximate concentration of 10µg/cm^2 on glass coverslips. The collagen was air dried onto the coverslips overnight and the coverslips washed three times with sterile distilled water then once with medium before use.

Preparation of Schwann cell precursor cultures

Schwann cell precursors were prepared essentially as described in Jessen et al. (1990). Sciatic nerves were dissected from embryos of varying ages, from E14 to E18. The nerves were cut into small pieces and incubated with 600µl enzyme cocktail solution at 37°C in 5% CO_2/95% air. Enzyme cocktail solution consists of Kreb's solution without calcium and magnesium (12mM NaCl, 4.7mM KCl, 1.2mM KH_2PO4, 11mM glucose, 1:50 amino acid solution (Gibco Life Technologies) buffered with $NaHCO_3$), with 3mg/ml collagenase (type 2; Worthington Biochemical Co., Freehold, N.J., USA), 1.2mg/ml hyaluronidase (Sigma) and 0.3mg/ml chick egg white trypsin inhibitor (Sigma). The tissue was incubated for 1 hr, triturated through a 1ml pipette tip then through a 200µl pipette tip, before being reincubated for a further 20 min, with trituration through a 200µl pipette tip after 10 min and at the end of the 20 min reincubation. The cell suspension was transferred to a centrifuge tube, the volume

made up to 10ml with DMEM containing 10% FCS (Advanced Protein Products, Brierley Hill, England), and centrifuged at 1000rpm for 10 min. In experiments described in Chapter 5, this was followed by a further wash with 10ml DM to remove any serum present. In Chapter 6, DM alone was used to make the volume to 5ml before centrifugation. The cells were resuspended in DM containing growth factors appropriate to the experiment (described in the text). After counting, the cells were plated out onto glass coverslips or plastic 4-well multidishes depending on the experiment. The drop size and number of cells depended on the type of assay to be undertaken, these details are given in the text. The cells were incubated for 3 hr at 37°C in 5% CO_2/95% air before topping up with medium required by the experiment.

K.R. Jessen performed the embryonic dissections for chapters 3-5, and many dissections for chapter 6.

Preparation of neonate Schwann cells

Sciatic nerves of newborn rat pups were dissected and the epineurial sheaths removed prior to dissociation in enzyme cocktail solution as detailed above. After digestion in enzyme for 1 hr at 37°C in 5% CO_2/95% air, the tissue was dissociated by sequential trituration through a 1ml pipette tip and a 200µl pipette tip. No reincubation with enzyme was necessary. The cell suspension was made up to 10ml with DM, centrifuged and resuspended in DM. The plating medium differed in the growth factors added, containing 1µM insulin (Sigma) (Chapters 3 and 4), either 1µM recombinant insulin (Sigma) or 100ng/ml IGF-1 (Kabi Pharmacia, Uppsala, Sweden) in Chapter 5 (as described in the text), and 1nM recombinant insulin in Chapter 6. Cells were counted and plated as described previously.

Survival assay

Following incubation of the cells at 37°C in 5% CO_2/95% air for three hours, 280µl of appropriate medium were added to each well. Three coverslips were incubated for a further 5 min, then fixed. Fixation was either for 20 min in 2% paraformaldehyde (BDH) with the coverslips mounted unstained on glass slides, or the cells were prefixed for 5 min with 2% paraformaldehyde in phosphate buffered saline (PBS) followed by labelling for either the p75 low affinity nerve growth factor receptor (p75LNGFr; a gift from Dr. E. Johnson Jr.) or L1 (Sweadner, 1983; a gift from Dr. A.

Furley). The remaining coverslips were cultured at 37°C and stained for p75LNGFr or L1 at a time point appropriate for the experiment. For the standard survival assay, the cells were fixed and labelled at 20 hr post-plating.

The number of cells that had attached and flattened at 3 hr and were positive for p75LNGFr (Chapters 3, 4, and 5) or L1 (Chapter 6) were taken to be the original starting population, survival was calculated as the proportion of these cells still alive after the culture period and expressed as a percentage.

Long term survival assays (Chapters 5 and 6)

The medium of long term cultures was replaced using fresh growth factor aliquots each day. As before, the number of cells surviving at the end of the culture period was calculated relative to the number of cells alive and flattened 3 hr post-plating.

Subculture and replating of long term cultures (Chapter 6)

Cells were cultured at an initial density of 12,000 cells per 50µl drop on PLL coated, laminin-coated plastic culture wells. To subculture the cells, the wells were rinsed briefly in versene (0.2% EDTA in PBS) and incubated for 4 min with 100µl versene and 40µl enzyme cocktail. The cells were triturated and transferred to a centrifuge tube that had been coated with defined medium. The cells were centrifuged at 1000rpm for 10 min at 4°C and resuspended in defined medium with 1nM recombinant insulin and 100ng/ml IGF-1. They were then plated at 2000 cells in 20µl drops on laminin-coated glass coverslips and incubated for 3 hr at 37°C in 5% CO_2/ 95% air. The wells were topped up with 300µl defined medium containing 1nM recombinant insulin and 100ng/ml IGF-1. In assays for the mitogenic effect of bFGF with the cAMP elevating agent forskolin, the medium also contained 3ng/ml bFGF and 5µM forskolin (Calbiochem-Novabiochem, San Diego, USA). Some coverslips were taken at this 3 hr point and stained for L1 or double-labelled for p75LNGFr and S100 (see below).

Growth factors

Epidermal growth factor (EGF) was supplied as a stock solution of 100µg/ml in DMEM with 1% FCS (a gift from Dr. F. Watt, ICRF); recombinant PDGFs AA and BB (both PeproTech Inc, NJ, USA) were reconstituted in 4mM acetic acid and further diluted in PBS to a stock concentration of 2µg/ml. Recombinant human Neu

differentiation factor-β2 (NDF-β2; Amgen, Thousand Oaks, CA, USA) was diluted in PBS with 0.5mg/ml BSA to a stock concentration of 5μg/ml. Recombinant human transforming growth factor-β1 (TGF-β1; Boehringer Mannheim, Lewes, England) and purified porcine TGF-β2 (R & D systems, Abingdon, England) were reconstituted in 4mM HCl with 10 mg/ml BSA to a stock dilution of 1μg/ml. Murine stem cell growth factor (SCF, mast cell growth factor, steel factor; a gift from Immunex, Seattle, USA) was supplied as a stock solution of 100μg/ml in 100mM Tris pH 7.4. Purified nerve growth factor (NGF; a gift from Dr. J. Winter, Sandoz Institute, UCL.) was supplied in medium at a concentration of approximately 5μg/ml. Recombinant human acidic fibroblast growth factor (aFGF, FGF-1; a gift from Dr M. Jaye, Rohrer Biotechnology) was supplied as a stock solution of 800μg/ml and was used from a substock of 8μg/ml made monthly in 1M NaCl in 10mM Tris pH7.4 and kept refrigerated. Recombinant human Kaposi's sarcoma fibroblast growth factor (K-FGF, FGF-4), stock concentration of 43μg/ml, and a fragment of this molecule, K-140, stock concentration of 41.5μg/ml were gifts from Dr. C. Basilico. Recombinant human endothelin-1 (ET-1; Cambridge Research Biochemicals) was dissolved in 0.1% acetic acid to a concentration of 1mM and then diluted to 10μM in L15 with 0.1% BSA. Recombinant endothelin-2 (ET-2) and endothelin-3 (ET-3) were were gifts from Dr. P. Milner, UCL, supplied at a concentration of 10μM in PBS.

Stock solutions of all other growth factors were made in L15 medium with 10mg/ml BSA. The sources of the other growth factors are as follows:

Recombinant human insulin-like growth factors I and II (IGFs I and II) were from Kabi Pharmacia, stock concentrations 10mg/ml. Basic fibroblast growth factor (bFGF) was from both PeproTech, and R & D systems, with a stock concentration of 2 μg/ml. Brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) were a gift from Dr. G. Dechant and Dr. Y. Barde, stock concentrations of 2 μg/ml. Ciliary neurotrophic factor (CNTF), stock concentration 100μg/ml, and NT-4, stock concentration 2 μg/ml were gifts from Regeneron, Tarrytown, USA. α-melanocyte stimulating hormone (α-MSH; Sigma) was at a stock concentration of 10μM. Recombinant murine leukaemia inhibitory factor (LIF) was a gift from Drs. L Grey and J. Heath, stock concentration 10μg/ml.

TPA (12-O-Tetradecanoylphorbol 13-acetate; Sigma) was at a stock of 2.5mM in ethanol and calcium ionophore A23187 (Sigma) was at a stock concentration of 100μM in ethanol. Heparin (Sigma) was kept at a stock concentration of 20μg/ml.

All growth factors were aliquoted into 10 or 20μl aliquots, (50 and 100μl aliquots in the case of IGFs 1 and 2), and stored refrigerated or frozen at –70°C as appropriate. Fresh aliquots were taken for each experiment and when changing the medium daily on long term cultures.

Neurone culture

Cultures of dorsal root ganglia neurones were prepared from newborn rats. The ganglia were removed, trimmed and incubated with 1.25mg/ml trypsin (Gibco Life Technologies) in Kreb's solution without calcium and magnesium, for 20 min at RT. An equal volume of DMEM containing 10% donor calf serum (CS; Sigma) was added and the ganglia dissociated by drawing the tissue through decreasing diameter needles (0.8mm, 0.6mm and 0.5mm) three times through each needle. Leaving the larger pieces of tissue behind, the cell suspension was centrifuged at 1000rpm for 10 min and the cells resuspended in DMEM containing 5% horse serum (HS, hybrimax; Sigma), 5% CS and 1μM insulin. The cell suspension was plated out on PLL-coated 35mm petri dishes (Nunc, Gibco Life Technologies) and NGF added at a final concentration of 50ng/ml.

To purify the cultures, 10μM cytosine arabinoside (Ara C; Sigma) was added for the first three days in culture, the cells were then switched to the same medium without Ara C for 2-3 days and back into Ara C containing medium for a further 4-5 days. The cells were then pulsed with Ara C in this medium as required to maintain optimally pure neuron cultures. These cultures contained between 800,000 and 1,200,000 neurones per petri dish, calculated by counting cells in a representative field and extrapolating this count to the area of the petri dish.

Neurone conditioned medium

Cultures of neurones were changed into defined medium containing 2.5% CS and 2.5% HS with 50ng/ml NGF for one day. They were then washed in defined medium and changed into 1.5ml defined medium, with or without NGF, for conditioning. After 2 days, the defined medium was collected and replaced with a further 1.5ml

defined medium, to be conditioned for a further 2 days before returning the cultures to DMEM 5% HS, 5% CS and 50ng/ml NGF.

The conditioned medium was either used fresh or stored frozen at −70°C in cryotubes (Nunc, Gibco Life Technologies) precoated with heat-treated BSA in PBS, (0.3% BSA in PBS heated to 80°C for 3 min), and washed in deionised water prior to use.

Neural crest culture

Culture of rat neural crest were carried out essentially as described by Smith-Thomas and Fawcett (1989) and Bannerman and Pleasure (1993). E11 embryos of 18-26 somites were used. The most caudal 6-8 somites and a small piece of contiguous non-segmented tail region were excised using fine needles. After trimming off accompanying tissue, the neural tubes were incubated in 0.1% collagenase in Kreb's solution without calcium and magnesium for 15 min on ice and then for 10 min at 37°C. The tissue was transferred to L15 medium containing 10% FCS and 1ng/ml bFGF on ice. The neural tubes were cleaned of surrounding somites by pipetting through a wide bore glass pipette and by teasing with fine needles. The cleaned tubes were transferred to defined medium without dexamethasone, containing 2% FCS, and 1ng/ml bFGF prior to plating in a drop of this medium on PLL-coated, fibronectin-coated coverslips. After 2 hr incubation at 37°C, the cultures were flooded with the same medium. Outgrowth of neural crest cells could be seen by 20 hr post-plating.

In some experiments to determine the survival requirements of neural crest, neural tubes were dissected in serum-free conditions using L15 medium or DM as described above but supplemented with 3ng/ml bFGF and 100ng/ml IGF-1. After culture for 20 hr the neural tubes were excised using fine needles and either the medium exchanged or the cells replated as described for Chapter 6. The medium used for survival contained 100ng/ml IGF-1, 1nM insulin and either 3ng/ml bFGF, 1.5ng/ml NDF or 10nM ET-1.

Immunohistochemistry

Prefixation of coverslips was performed in the culture wells by addition of an equal volume of 4% paraformaldehyde (final paraformaldehyde concentration 2%). Fixation was for 5 min at RT, after which the coverslips were removed from the culture wells, washed and incubated with the relevant antibodies.

Antibodies used on live cell cultures or cells prefixed for 5 min with 2% paraformaldehyde were diluted in modified Eagle's medium (MEM; Gibco Life Technologies) containing 15mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, Sigma) (MEM-H) and 10% CS. Washes between antibody steps were in MEM-H.

Antibodies used on fixed cell cultures were diluted in antibody diluting solution (PBS containing 10% CS, 0.1M lysine (Sigma) and 0.02% sodium azide (Sigma)). Post-fixing washes were in PBS.

After antibody labelling and fixation, all cultures were mounted on glass slides in Citifluor anti-fade medium (UKC, Canterbury, UK) and sealed.

Conjugated antibodies

Fluorescein-conjugated goat anti-mouse and goat anti-rabbit Igs, and tetramethyl rhodamine-conjugated goat anti-mouse and goat anti-rabbit Igs were from Cappel, Organon Teknika, Durham, USA. These second layers were preabsorbed against immobilized rabbit and mouse Igs to remove cross-reacting antibodies as described below. Biotin-conjugated sheep anti-mouse and sheep anti-rabbit antibodies, and streptavidin-fluorescein were from Amersham Life Sciences, Amersham, England. These were used without preabsorption at a dilution of 1:100.

Removal of cross-reacting antibodies in second layers

Sepharose-linked immunoglobulins were packed into two columns of 0.5ml bed volume on nylon wool. The columns were washed in PBS and the relevant conjugated antibody was run through the columns in succession, using a small amount of PBS to elute the last of the unbound antibody. The columns were regenerated with 15ml 5M magnesium chloride followed by 7ml PBS. Sodium azide was added to the purified antibody at a final concentration of 0.02% prior to storage. The titre of each batch of antibody was tested before use and adjusted so that fluorescein-conjugated anti-mouse Igs and rhodamine-conjugated anti-rabbit Igs were used at dilutions of 1:100, and rhodamine-conjugated anti-mouse Igs and fluoresein-conjugated anti-rabbit Igs were used at dilutions of 1:200.

BrdU incorporation and visualization

Cells were exposed to either 20μM BrdU (Boehringer Mannheim) for the last 1.5 hr of culture or to 10μM BrdU for the whole culture period. They were then prefixed for 5 min in 2% paraformaldehyde and stained with either p75LNGFr or L1 as detailed below. Fixation in methanol at –20°C for 10 min was followed by incubation with 2N hydrochloric acid (HCl) for 20 min at RT. Following extensive washes in PBS, the cells were treated with 0.1M sodium borate, pH 8.5, for 10 min to neutralize any remaining acid. The coverslips were drained and incubated with anti-BrdU antibody (Gratzner, 1982; a gift from Dr D. Mason) diluted 1:100 in PBS containing 0.1% triton-X100 (BDH) for 1 hr at RT. The anti-BrdU antibody was visualized with rhodamine-conjugated goat-anti-mouse immunoglobulins diluted 1:200 in PBS containing 0.1% triton-X100 for 30 min before washing and mounting on slides.

p75LNGFr

The monoclonal antibody 192-IgG, against the p75LNGFr was a gift from Dr. E Johnson Jr. (Taniuchi et al., 1986), was used at 1:100 from hybridoma supernatant, or 1:500 when in the form of ascites. The monoclonal antibody 217c, also recognising p75LNGFr (Ferrari et al., 1991) was used in the form of culture supernatant at 1:200. The antibody was incubated on prefixed cultures for 30 min at RT.

L1

The monoclonal antibody ASCS4, against L1 (Sweadner, 1983; a gift from Dr. A. Furley) was used in the form of neat hybridoma supernatant or at a 1:1 dilution with MEM-H 10%CS on prefixed cells. Incubation was for 1 hr at RT, followed by biotin-conjugated anti-mouse Igs for 30 min and streptavidin-fluorescein for 15 min.

Rabbit anti-L1 (a gift from Dr. M. Schachner; Rathjen and Schachner, 1984) was used at a dilution of 1:200 for 30 min on live cells without prefixation. This polyclonal antibody was used exclusively for Chapter 3 and is not the same as that used throughout the rest of this thesis.

S100

Following labelling for p75LNGFr, the cultures were fixed with 4% paraformaldehyde for 20 min and treated with methanol at –20°C for 10 min. To remove non-specific background, the coverslips were incubated with 3% gelatin (type 2, bovine skin,

Sigma) in PBS for 30 min, then incubated with anti-S100 (Dakopatts, Copenhagen, Denmark) diluted 1:10,000 overnight at RT. The label was visualized using sheep anti-rabbit biotin for 30 min, followed by streptavidin-fluorescein for 15 min. Control coverslips for this label omitted the anti-S100 antibody and were incubated in antibody diluting solution alone.

GAP-43

Cultures were fixed in 4% paraformaldehyde in PBS for 20 min, followed by methanol at –20°C for 10 min. Rabbit anti-GAP-43 antibody (Curtis et al., 1991; a gift from Dr. R. Curtis) was used at a final dilution of 1:1000 for 30 min.

Vimentin

Cultures were fixed in 95% ethanol, 5% acetic acid (acid alcohol), for 15 min, followed by 0.3% triton-X100 in PBS for 20 min. A mouse monoclonal antibody to vimentin (Boehringer Mannheim) was used at 1:100 for 30 min.

NCAM

Rabbit anti-NCAM (Gennarini et al., 1984) was used at a dilution of 1:500 for 30 min on cells after prefixation of cultures with 2% paraformaldehyde for 5 min.

A5E3

The mouse monoclonal antibody A5E3 in the form of ascites (Mirsky et al., 1985) was used at a dilution of 1:500 on live cells for 30 min.

Ran-2

Purified mouse monoclonal antibody Ran-2 (Bartlett et al., 1981) was used at a dilution of 1:50 for 30 min on live cells.

04

Mouse monoclonal antibody 04 (Sommer and Schachner, 1981) in the form of hybridoma supernatant was used at a dilution of 1:1 for 30 min on live cells.

Galactocerebroside

Mouse anti-GalC, a monoclonal antibody in the form of ascites (Ranscht et al., 1982), was used at a dilution of 1:100 on live cells for 30 min.

GFAP

Cultures were fixed in acid alcohol for 15 min, followed by treatment with 0.3%

triton-X100 in PBS for 20 min. Rabbit antiserum to glial fibrillary acidic protein (GFAP; Dakopatts) was used at a dilution of 1:100 for 30 min.

Laminin

Rabbit polyclonal anti-laminin (a gift from Dr. R. Timpl) was used at dilution of 1:500 on live cells cultured on fibronectin-coated coverslips.

Rat 401/Nestin

Mouse monoclonal antibody (Friedman et al., 1990) in the form of hybridoma supernatant, was used neat on cells that had been fixed in 4% paraformaldehyde for 20 min and then treated with methanol for 10 min at ¯20°C.

P_0

Rabbit polyclonal anti-P_0 was generated in the laboratory essentially as described by Brockes et al., 1980 (Morgan et al., 1994). The antiserum was purified by incubating with chloroform-extracted newborn rat skin for 48 hr, followed by precipitation with caprylic acid, then 40% ammonium sulphate.

Cells were fixed in 2N HCl for 15 min, washed extensively in PBS then further neutralized with 0.1M sodium borate solution for 10 min. The cells were then blocked for 2 hr with antibody diluting solution, washed and incubated in anti-P_0. The antibody was used at a dilution of 1:1000 for 2 hr to detect high levels of protein expression found in myelinating Schwann cells, and at 1:200 overnight at 4°C to detect low levels. Both concentrations were visualized using fluorescein-conjugated anti-rabbit Igs.

Myelin basic protein, (MBP)

Monoclonal antibody to MBP (Boehringer Mannheim) was used at a concentration of 1:200 overnight on cells fixed in 4% paraformaldehyde for 20 min.

Hoechst staining

A stock solution of 1mg/ml Hoechst dye H33258 (bisBenzimide; Sigma) in water, was diluted 1:1000 in PBS and applied to fixed cell cultures for 10 min prior to mounting and viewing with Hoechst optics.

Labelling of dying cells that had detached from the monolayers with Hoechst dye was performed as follows: the cells were spun for 10 min at 600rpm, and resuspended in 1

volume of PBS. They were then fixed by addition of 2 volumes of 4% paraformaldehyde for 10-15 min. The cells were resuspended in this fixing mixture and transferred dropwise to a washed nitrocellulose millipore filter (Schleicher and Schuell, Dassel, Germany) using absorbant paper to draw the liquid through the filter. The filter containing the cells was then washed twice with PBS, transferred to a petri dish and stained with Hoechst dye 1:1000 in PBS for 20 min.

Quantification

Most results are based on three separate experiments, using duplicate or triplicate coverslips (occasionally quadruplicate coverslips). Where the results are from only one or two experiments this is stated in the text.

At least 300 cells per coverslip were counted for BrdU incorporation, and at least 250 cells per coverslip were counted for S100 expression.

Survival for all experiments is expressed as 'percentage survival', referring to the number of cells surviving at the experimental timepoint relative to the number of cells alive and flattened at 3 hr post-plating.

Error bars in the graphs indicate SEM, or SD in the case of experiments with only two repetitions.

Microscopy

All cultures were viewed using a Zeiss microscope and x 20, x 40 dry or x 63 oil immersion phase-contrast lenses. Epi-illumination was used with rhodamine, fluorescein or Hoechst filters.

DNA fragmentation assay

A total of 400,000-500,000 cells were plated at a concentration of 50,000 cells per well on PLL coated, laminin-coated culture wells, in defined medium with 1μM insulin. After 3 hr, the medium was removed and replaced with either NCM or defined medium with 1μM insulin. At 5 hr or 7 hr post-plating, floating cells were harvested by centrifugation of the culture medium. Cells still attached to the substrate were washed briefly in PBS and lysed in 100μl lysis buffer (total volume), together with the cells harvested from the culture medium. The lysis buffer contained 10mM Tris (pH 8), 100mM NaCl, 1mM EDTA and 0.5% sodium dodecyl sulphate (SDS).

The samples were treated essentially as described by Davis et al (1986). Proteinase K (Fluka, Gillingham, England) was added to the lysates to a final concentration of 200µg/ml which were then incubated for 3-18 hr at 50°C. After cooling to RT, RNase A (Gibco Life Technologies) was added to a final concentration of 200µg/ml and incubated for 30 min at 37°C. The samples were extracted twice with phenol : chloroform (1:1) (both BDH) and the aqueous layer containing the DNA was taken for electrophoresis.

Gel electrophoresis

The samples were run on 1.7% agarose gel (Pharmacia, Milton Keynes, England) in 1x TBE (0.09M Tris borate, 0.002M EDTA) with 0.5µg/ml ethidium bromide (Sigma) in the gel and with 1x TBE as running buffer. The sample was diluted 1:6 in loading buffer (30% glycerol in water, 0.25% bromophenol blue (Sigma)) and ethidium bromide was added to the sample (final concentration 40µg/ml) before loading 20µl per lane on the gel which was then run at 100mV for 1 hr. The gel was destained by soaking in deionised water, with several changes, for 1 hr. The DNA was visualized on a transilluminator and photographed using polaroid film at f16 for 1 second.

CHAPTER 3

ISOLATION AND CHARACTERISATION OF THE SCHWANN CELL PRECURSOR

INTRODUCTION

Rat Schwann cells have been studied in detail both in the perinatal and adult animal (Mirsky et al., 1990; Jessen et al., 1990; Morgan et al., 1991; Gould et al., 1992; Jessen and Mirsky, 1993; Stewart et al., 1993). Schwann cells from nerves of late embryonic rats (E18 and older) have a phenotype similar to that of cultured Schwann cells from later animals (reviewed in Mirsky and Jessen, 1990). There are few data available, however, about glial cells associated with nerves early in development. This chapter is a study of the glial cells associated with the sciatic nerve of early embryonic rats (and brachial plexus in the case of E14 rats) and defines the previously undescribed phenotype of the glial cells, Schwann cell precursors, associated with the nerves at E14-15. To understand the *in vivo* environment of these cells and the factors that may influence their development, there follows a description of the development of the peripheral nerve, in particular the rat sciatic nerve.

Neuronal development and limb innervation

The sciatic nerve is a mixed nerve with both motor fibres from the ventral spinal cord and sensory fibres from the DRG, originating from the lumbar spinal segments L4-L6. Neural crest cells that form the rat DRG begin to condense in the anterior half of the adjacent somite (or that of the more caudal somite) between E11 and E13 (Angulo, 1951; Sobkowicz et al., 1973; Lawson et al., 1974). The development of the DRG and the differentiation of the neurones within the ganglia proceeds in a rostro-caudal manner with the DRG of the cervical region developing ahead of those in the lumbar region. In the rat lumbar ganglia, some large neurones stop dividing at E11 and start to differentiate. The peak of this differentiation is seen at E12 for large ventrolateral neurones and E13 for the smaller dorsomedial neurones with most mitosis complete by E15 (Lawson et al., 1974).

At the lumbar level in the rat embryo, the motor neurones start to project axons from the ventral spinal cord towards their target tissue at E11. Axons of the DRG neurones start to grow out from the DRG at this time and join the motor axons to form loose bundles of nerves at E12 (Reynolds et al., 1991). These spinal nerves grow into the base of the hindlimb around E12, where they form a plexus of dense nerve bundles with axons projecting out into the surrounding tissue from the growing tip of the nerve

(Reynolds et al., 1991). A day later, the nerves are still tightly bundled and only project a short way into the developing hindlimb. During this time, the axons in the plexus defasciculate and reorganise, a process that has also been described in the chick hind limb (reviewed in Scott, 1987; Tosney and Landmesser, 1985). The nerve enters the proximal hindlimb at early E14; most axons at this time are still bundled in a plexus but the first branches are apparent, directed outward toward the skin (Reynolds et al., 1991). By E15 the nerves extend into two thirds of the developing limb and have started to branch into the developing muscles in the proximal limb. Increased branching of the nerve bundles is observed over the next few days and innervation of the most distal part of the hindlimb is detected by E19. Nerve terminals within the muscles are first apparent at E17, the branching of the terminals increases with time until fine sprays of immature terminals are apparent in all muscle nerves by E21. Mature endplates are seen in the muscle from the second postnatal day (P2), although they are associated with polyinnervation. Over the next two weeks, the terminals mature and synapses are eliminated so that a single axon is associated with each endplate (Reynolds et al, 1991).

Intitiation of electrical activity

In the rat embryo, spontaneous electrical activity can be detected in the lumbar DRG neurones from E16, peaking between E18 and E19 (Fitzgerald, 1987). The first action potentials to be recorded in the dorsal horn neurones, in response to stimulation of the skin of the hind limb, occur at E19, although electrical stimulation can produce a response as early as E17 (Fitzgerald, 1990). The peripheral nerves innervating the rat intercostal muscles have been shown to be excitable from E13 onward, a day earlier than the formation of nerve-muscle contacts in this region (Ziskind-Conheim, 1988). Action potentials can be recorded in these nerves prior to myelination, with the conduction velocity increasing as the axon diameter increases. The initiation of electrical activity in the developing nerves correlates with development of spontaneous movement (E15 forelimb and E16 hindlimb) and reflexes (E16 forelimb and E17-18 hindlimb) (Narayanan et al., 1970).

Neural crest origin of peripheral nerve glial cells

In an early experiment on neural crest, Harrison (1924) showed in *Rana* (frog) that removal of the dorsal part of the neural tube prior to the migration of neural crest

resulted in a lack of DRG formation and an absence of sheath cells from the motor axons that projected into the periphery. He observed that, in normal embryos, the most distal sections of the axons are devoid of associated cells but, in the proximal sections, spindle-shaped sheath cells are closely associated with the axons. These cells migrate in a proximo-distal direction along the axons, except when they encounter the ventral roots, where migration appears to be directed along the roots toward the developing spinal cord.

This close association of sheath (or glial) cells with developing axons and their migration along the nerves has been confirmed in more recent studies in the tadpole tail fin (Billings-Gagliardi et al., 1974), and chick limb (Weston, 1963; Loring and Erickson, 1987; Dahm and Landmesser, 1988; Carpenter and Hollyday, 1992a, 1992b). In the chick, neural crest cells comigrate with developing spinal nerves, always in close association with the axons, but are absent from the most distal portion of the growing axons (Dahm and Landmesser, 1988; Carpenter and Hollyday, 1992b) a result confirmed in mouse (Bogusch, 1992). These cells are initially distributed in uneven groups along the axons, but with increased developmental age, the entire surface of the nerve bundles is ensheathed (Dahm and Landmesser, 1988). A few studies have indicated that Schwann cell precursors migrate ahead of the axons and may act as pathfinding glia (Noakes and Bennett, 1987; Serbedzija et al., 1990; Haninec and Dubový, 1992), but in Splotch mutant mice, where Schwann cells and DRG neurones are absent from lumbar regions due to failure of neurulation, motor neurones grow into the periphery with normal patterns of innervation, albeit at slower rates (Grim et al., 1992).

There is some evidence in the chick embryo that cells associated with the proximal ventral roots are derived from the ventral neural tube and are not neural crest in origin (Weston, 1963; Loring and Erickson, 1987; Lunn et al., 1987). Other studies have shown that the glial cells that populate the ventral roots are of neural crest origin (Carpenter and Hollyday, 1992a; Bhattacharyya et al., 1994) and it is possible that the cells in this region of the developing nerve are a mixture of neural tube and neural crest cells.

In the chick, removal of the ventral neural tube just prior to motor axon outgrowth and neural crest migration does not prevent neural crest migration to the ventral root

region of the embryo (Bhattacharyya et al., 1994). The loss of axons, however, prevents the arrest of the neural crest cells in this area, and results in the complete absence of Schwann cells in this region at later developmental stages. No increase in cell death is observed in this region in the absence of motor axons suggesting that the cells continue to migrate, possibly to associate with the axons of the developing sensory neurones.

Glial-axonal relationships in the developing nerve

Ultrastructural studies reveal that, in the rat, the glial cells associated with early developing nerves, at E14, are found around the perimeter of nerve axon bundles, separating the axons from the surrounding mesenchyme (Peters and Muir, 1959; Jessen et al., 1994), and many nuclei are present within the bundles in the larger branches of the nerve (Jessen et al., 1994; Hashimoto and Jessen, personal communication). These cells have fine processes that extend into the bundles of tightly packed, small diameter axons, each glial cell being in contact with many axons; the slender processes of the glial cells separate the axons into domains. No basal lamina is found in the nerve at this time.

Although there is extensive proliferation of the glial cells in the nerve at E14, there is a dramatic peak of proliferation of Schwann cells, between E17 and E21, the day of birth (Peters and Muir, 1959; Stewart et al., 1993). As a result of glial cell proliferation and neuronal death, the number of glial cells within the nerve increases with concommitant decrease in the number of axons associated with each glial cell (Peters and Muir, 1959). Basal lamina is first detected in the nerve at E16-17 (Bignami et al., 1984).

By birth, some Schwann cells are associated with single, large diameter axons, but many Schwann cells enfold several smaller diameter axons. Maturation of the nerve takes place over the next five to six weeks, by which time single, large diameter axons are found in association with myelin-forming Schwann cells and groups of small diameter axons are ensheathed by non-myelin-forming Schwann cells (Webster and Favilla, 1984; Jessen and Mirsky, 1990). Myelination of axons in a 1:1 relationship with Schwann cells begins at birth and is preceeded by withdrawal of the cells from the cell cycle (Stewart et al., 1993).

This chapter describes the first stages of the characterization of the rat Schwann cell precursor, a cell that can be isolated from the embryonic sciatic nerve at E14. Using embryonic nerves from different ages, the changes in morphology, expression of the Schwann cell marker S100 and survival ability in the absence exogenous growth factors of these cells during embryonic development will be described.

RESULTS

Glial cells isolated from early embryonic nerve exhibit morphological and phenotypic differences from perinatal Schwann cells

Schwann cells isolated from perinatal nerves (E17 to newborn) expressed both the low affinity p75 nerve growth factor receptor (p75LNGFr) and the calcium-binding protein S100 (Fig 3.1, D, E, F). When limb nerves from E14 and E15 embryos were dissociated and the cell suspension plated on laminin-coated coverslips, 95% ± 4 (SEM, n=4) of the cells were found to express p75LNGFr, as determined by the antibodies 192-IgG (Fig 3.1 B) and 217c (Ran-1) (not shown). However, the Ca^{2+}-binding protein S100 that is ubiquitously expressed in perinatal and adult peripheral glia was not detected in the embryonic Schwann cells (Fig 3.1 C) using immunohistochemical techniques that stained cells from E17 and older nerves (Fig 3.1 F).

Morphological differences between cells isolated from E14 nerves and perinatal nerves were obvious from about 3 hr in culture. The early glial cells, plated onto laminin-coated coverslips, exhibited an epithelial morphology, forming flattened groups of cells with extensive contacts between the cells (Fig 3.2 A). In contrast, cells from newborn nerves under the same conditions exhibited an elongated bi- or tripolar morphology with little contact between the cells (Fig 3.2 B). An intermediate morphology was seen in cells from late embryonic nerves (E17 and E18, Fig. 3.2 C).

Expression of S100 is developmentally regulated in peripheral nerve glial cells

Limb nerves from embryonic rats of different developmental ages, E14-E18 and newborn, were dissociated and plated out on laminin-coated coverslips. At 3 hr post-plating, the cells were stained for p75LNGFr and S100. The percentage of $p75LNGFr^+$ cells that also expressed S100 is shown in Fig. 3.3.

No S100 expression could be detected in cultures from E14 and E15 nerves. The expression of S100 was first seen in the nucleus and rapidly spread to the surrounding cytoplasm at E16. By E17 the majority of $p75LNGFr^+$ cells also expressed cytoplasmic S100. The level of S100 immunolabelling seen in the cytoplasm increased from E17 through to birth, suggesting that the level of S100 protein continued to increase in these cells.

Culture in defined medium distinguishes embryonic glia from newborn Schwann cells

Dissociated nerves from E14 rats were plated out in defined medium with 1μM insulin, 4000 cells per 20μl drop, on laminin-coated coverslips. At 3 hr, the coverslips were either fixed and stained for p75LNGFr or topped up with defined medium containing insulin and cultured for a further 17 hr before staining for p75LNGFr. Parallel experiments with nerves from newborn rats were performed under the same conditions.

At 3 hr, cultures from both E14 and newborn nerves showed similar numbers of cells that had attached and flattened, and appeared to be healthy; for newborn cultures this was 2095 ± 125 cells (SEM, n=4) and for E14 cultures 2523 ± 230 cells (SEM, n=10) (Fig 3.5 A).

A dramatic difference was observed at 20 hr: the cultures of newborn Schwann cells contained bi- and tripolar cells with almost all cells present at 3 hr surviving to 20 hr under these culture conditions (Fig 3.4). In the cultures of E14 cells, however, there were very few cells left alive (Fig 3.4). These cultures contained mainly cell debris and rounded, dead or dying cells (Fig 3.5 B). The manner of this death will be the subject of Chapter 4.

When tissue from the surrounding mesenchyme was cultured in the same way as the embryonic nerves, many cells survived cultured in defined medium. The surviving cells looked like early fibroblasts, with short bi- or tripolar processes; cell-cell contact was limited (not shown). Of these cells, less than 5% expressed levels of p75LNGFr seen in the cultures of E14 nerve.

Developmental age determines survival ability in defined medium

Using similar procedures to those described above, limb nerves from newborn rats and embryonic rats of different ages (E14-E18) were dissociated and cultured for 3 hr and 20 hr in defined medium with 1μM insulin. The number of $p75LNGFr^+$ cells surviving at 20 hr was plotted as a percentage of the cells that had flattened at 3 hr (Fig 3.6).

At E14 and E15, less than five percent of the $p75LNGFr^+$ cells survived to 20 hr. The ability to survive in defined medium began to appear in cells isolated from E16 nerves;

about 15% of the cells survived to 20 hr. By E17, this number had risen to approximately 90%. At E18, the number of cells surviving at 20 hr was equal to that seen with Schwann cells isolated from newborn rat nerves.

Plating density does not affect survival of glial cells from E14 cultures

To determine whether the plating density affected the survival of E14 cells, cells were dissociated and plated at different densities in defined medium containing 1μM insulin. 1,000, 4,000 and 16,000 cells were plated in 20μl drops on laminin-coated coverslips and the number of $p75LNGFr^+$ cells surviving at 20 hr were counted (Table 3.1).

No difference in survival was detected in the different plating densities; essentially the same proportion of cells died even at the highest density. Thus, it appears that these early glial cells do not produce autocrine growth factor(s) that promote their survival.

Cell division does not account for the increased number of cells surviving with increased developmental age

To determine whether division of a subpopulation of the cells in culture was contributing to the number of cells surviving at 20 hr, cells were dissociated from E17 and newborn nerves and plated, as described previously, in defined medium with 1μM insulin. 20μM BrdU was added for the last 1.5 hr of the 20 hr culture and the cultures were then stained for p75LNGFr and BrdU incorporation.

In three separate experiments, with triplicate coverslips, the percentage of $p75LNGFr^+$ cells that had incorporated BrdU into their DNA in E17 cultures was 1.0, 0.5 and 0, and in newborn cultures, 1.0, 0.6 and 0.9 (both averages less than 1%).

Neurone-conditioned medium can prevent the death of E14 glial cells

Since the E14 glial cells do not appear to produce an autocrine growth factor under defined conditions *in vitro*, and since *in vivo* they are in intimate contact with axons of the growing nerve, defined medium conditioned by DRG neurones was used to see if it could support the early glial cells in culture.

Defined medium containing 1μM insulin was conditioned by purified cultures of DRG neurones for 2 days. E14 cells, plated in defined medium with insulin, were topped up at 3 hr with neurone-conditioned medium (NCM) and cultured for a further 17 hr.

At 20 hr, approximately 80% of the p75LNGFr$^+$ cells were still alive (Fig 3.5 C). The presence or absence of 50ng/ml of NGF in the medium prior to conditioning made only a small difference to the ability of the conditioned medium to promote survival: NCM without NGF produced survival that was 90% ± 2 (SEM, n=2) of that seen in the presence of NGF. Defined medium with 1μM insulin and 50ng/ml NGF did not rescue E14 cells when not conditioned by neurones (see Chapter 5, table 5.1).

To determine whether the NCM stimulated DNA synthesis in the rescued cells, 20μM BrdU was added to the E14 cells for the last 1.5 hr of culture. The cells labelled for both p75LNGFr$^+$ and BrdU at 20 hr were counted. Using triplicate coverslips, the percentage of p75LNGFr$^+$ cells that had incorporated BrdU was 1.0% ± 1 (SEM, n=3).

The molecular phenotype of E14 glial cells

As described previously, the molecular phenotype of Schwann cells from late embryonic nerves is well known (reviewed in Mirsky and Jessen, 1990). Immunohistochemistry was used to establish the similarities and differences between the glial cells from E14 nerves and perinatal Schwann cells.

Cultures of E14 cells were established on laminin-coated coverslips (except for staining for laminin expression where fibronectin was used as a substrate). Immunolabelling was performed at 3 hr and after 18 hr in NCM. No difference was seen between cells under the different conditions indicating that the phenotype was essentially unchanged after short-term culture.

More than 90% of cells expressed the adhesion molecules N-CAM and L1, the surface antigens A5E3 and Ran-2, , and the intermediate filament protein vimentin (Fig 3.7). Both 192-IgG and 217c antibodies against p75LNGFr were bound by 95% of the cells. Using the rat 401 antibody to nestin, 95% of the cells were positive; weak surface labelling for the ECM molecule laminin could also be detected.

As described earlier, S100 protein was undetectable in these cells, as was the intermediate filament glial fibrillary acidic protein (GFAP). The surface antigen 04 and the lipid galactocerebroside (GalC) were absent from the cells and the major Schwann cell myelin protein MBP was not detected. (Table 3.2). High levels of P_0, comparable to those found in myelinating cells were not observed in the E14 cells.

However, low levels of P_0 expression could be detected specifically in the E14 glial cells when high levels of antibody were used (not shown).

Are E14 glial cells migrating neural crest cells?

The morphology of the E14 cells is strikingly similar to that of rat neural crest cells in culture.* To determine whether these cells were in fact neural crest cells, cultures of rat E11 neural tubes were made and the migrating crest cells stained after 2 days in culture, in parallel with cells from E14 nerve after 20 hr culture in NCM.

Both cultures were double-labelled with antibodies to p75LNGFr and one of the following antibodies: N-CAM, L1, A5E3 or Ran-2. Both E14 glial cells and $p75LNGFr^+$ neural crest cells expressed N-CAM, L1, A5E3 and Ran-2 (Table 3.2). However, a difference between precursors and neural crest cells was observed when the cells were labelled for the membrane phosphoprotein GAP-43. More than 95% of the E14 glial cells were positive for this antigen but it was undetectable on $p75LNGFr^+$ neural crest cells. To confirm that GAP-43 expression was not due to differences in culture conditions, both neural crest cells and E14 glial cells were cultured in identical conditions (defined medium without dexamethasone, with 2% FCS and 1ng/ml bFGF). Again, GAP-43 was observed in E14 cells but not in neural crest cells (Fig 3.8).

To determine whether there were differences in survival requirements for the neural crest and precursors, neural tubes were explanted as described in Chapter 2, but using 3ng/ml bFGF and 100ng/ml IGF-1 instead of bFGF and serum. After 20 hr the neural tubes were excised and the neural crest either transferred into medium containing the growth factors to be tested, or the cells were replated as for Chapter 6, with addition of the same growth factors. Combinations of growth factors that support long-term precursor survival, namely 1.5ng/ml NDFβ-2 and 100ng.ml IGF-1, and 1nM ET-1 with 100ng/ml IGF-1 (see Chapter 6) failed to support neural crest survival beyond 20 hr after tube removal (data not shown).

*(Smith-Thomas and Fawcett, 1989)

Table 3.1 The effect of density on cell survival

Cells were plated in DM containing 1μM insulin in 20μl drops on laminin-coated coverslips. The cells were stained for p75LNGFr at 20 hr post-plating. Increasing the cell density from 1,000 cells/ 20μl to 16,000 cells/ 20μl did not increase the proportion of cells surviving

Table 3.1 Effect of density on cell survival

Experiment	Number of cells plated	$p75LNGFr^+$ cells at 20 hr
1.	1000	6
	4000	9
	16000	34
2.	1000	7
	4000	36
	16000	108
3.	1000	10
	4000	15
	16000	50

Number of cells plated	Mean survival at 20 hr	SEM (n=3)	% of total cells plated
1000	8	2	> 1
4000	20	10	> 1
16000	64	28	> 1

Table 3.2 The molecular phenotype of E14 glial cells

Cells isolated from E14 nerves were immunolabelled after 3 hr and 18-20 hr in culture in NCM as described in Chapter 2. Neural crest cells were immunolabelled after 20 hr in culture with bFGF and 2%FCS. In all cases where cells were positive, more than 90% of the cells were labelled.

Table 3.2. Molecular phenotype of E14 glial cells

Antigen	E14 glial cells	E11 neural crest cells
S100	–	–
p75LNGFr (192 Ig)	+	+
p75LNGFr (217c)	+	+
N-CAM	+	+
L1	+	+
A5E3	+	+
Ran-2	+	+
Laminin	+	n.d.
GAP-43	+	–
Vimentin	+	n.d.
Nestin	+	n.d.
GFAP	–	n.d.
04	–	n.d.
GalC	–	n.d.
P_0	–	n.d.
MBP	–	n.d.

(n.d. = not determined.) The phenotypical analysis was performed on two sets of cultures for both E14 and neural crest cells.

Table 3.3 The molecular phenotype of Schwann cells

The antigen expression for myelinating, non-myelinating and short term cultured Schwann cells is as reported in Gould et al., 1992 (also described in Table 1.1). Antigens in section 1) are expressed by mainly non-myelinating cells, 2) expressed by both myelinating and non-myelinating cells and 3) by myelinating cells. Antigen expression for precursors is as described in table 3.2

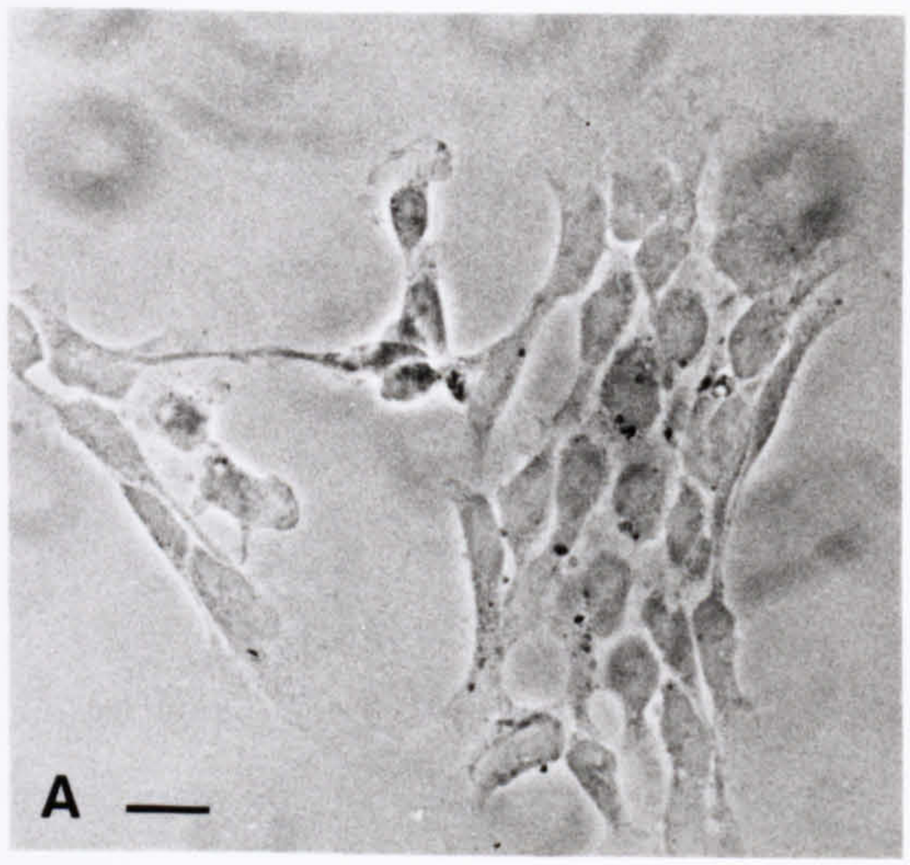
[a] Present mainly at nodes of Ranvier.

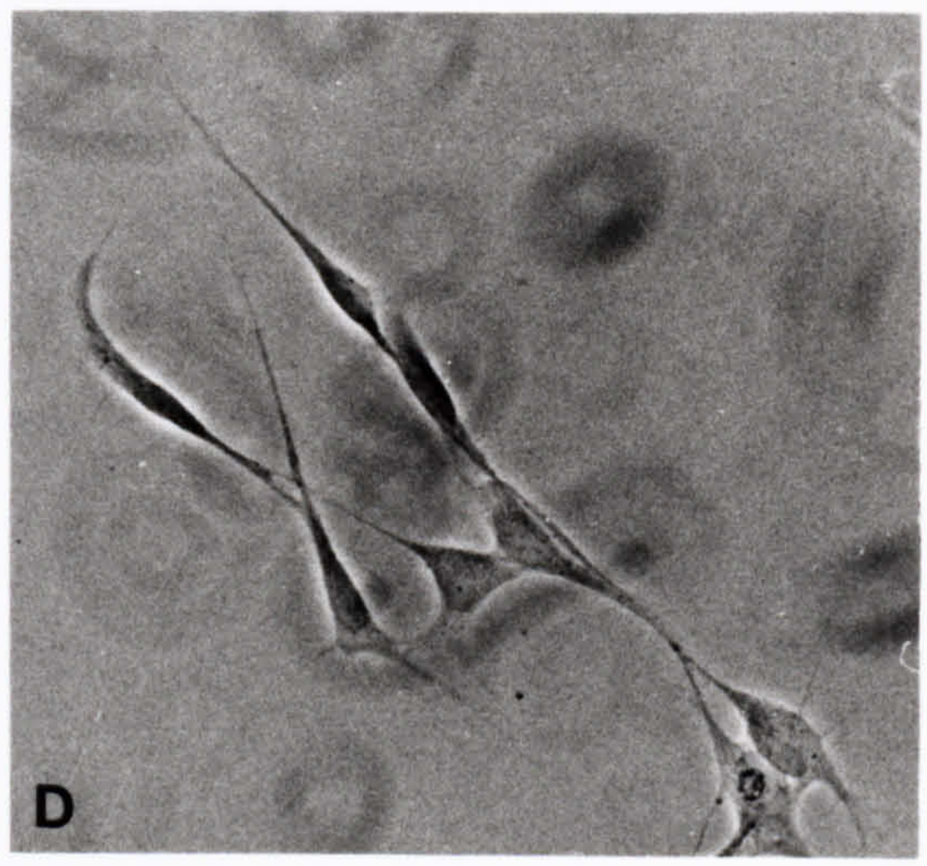
Table 3.3 The molecular phenotype of Schwann cells

Antigen	Myelinating Schwann cells	Non-myelinating Schwann cells	Short term cultured Schwann cells	Schwann cell precursors
1) p75LNGFr	−	+	+	+
N-CAM	−	+	+	+
L1	±[a]	+	+	+
A5E3	−	+	+	+
Ran-2	−	+	+	+
GAP-43	−	+	±	+
GFAP	−	+	+	+
2) Vimentin	+	+	+	+
Nestin	+	+	+	+
S100	+	+	+	−
04	+	+	−	−
GalC	+	+	−	−
3) P_0	+	−	−	−
MBP	+	−	−	−

Figure 3.1 Comparison of p75LNGFr and S100 expression between E14 and perinatal Schwann cells

Schwann cells isolated from E17 rats and cultured for 3 hr in defined medium are shown in phase contrast in (D) and express the p75LNGFr as recognised by the antibody 192 Ig (E); these cells also express S100 (F). Nerves from E14 rats cultured under the same conditions are shown in phase contrast in (A). They also express p75LNGFr at 3 hr (B) but do not express S100 (C). Bar = 20μm.


A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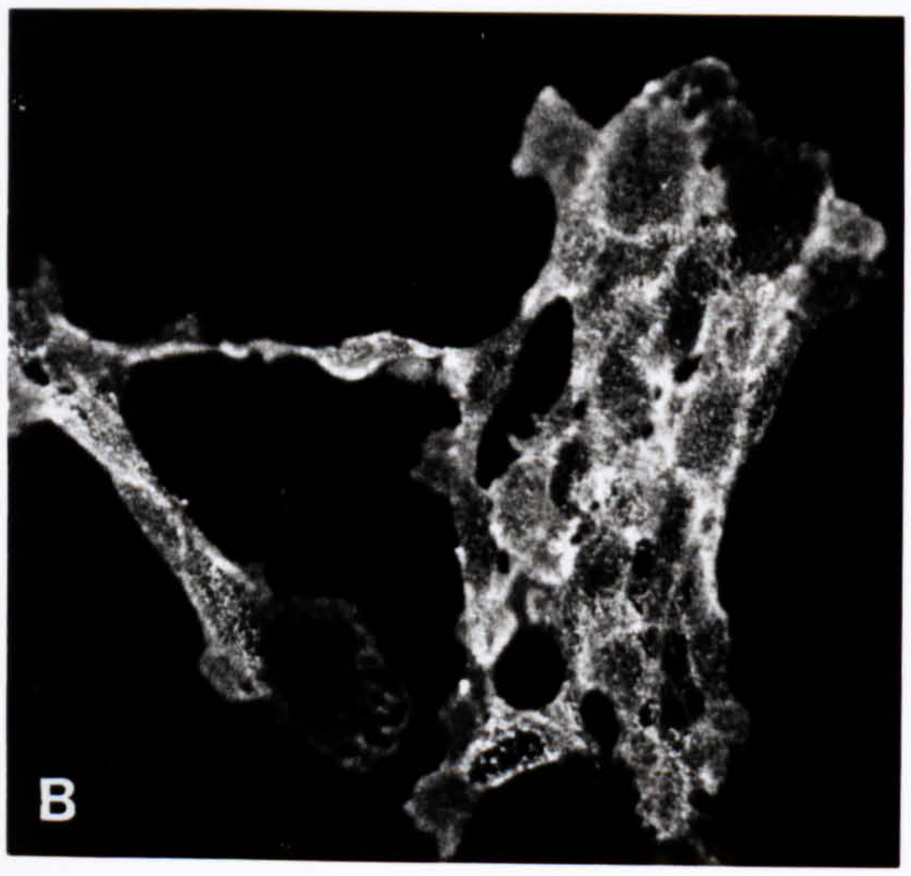
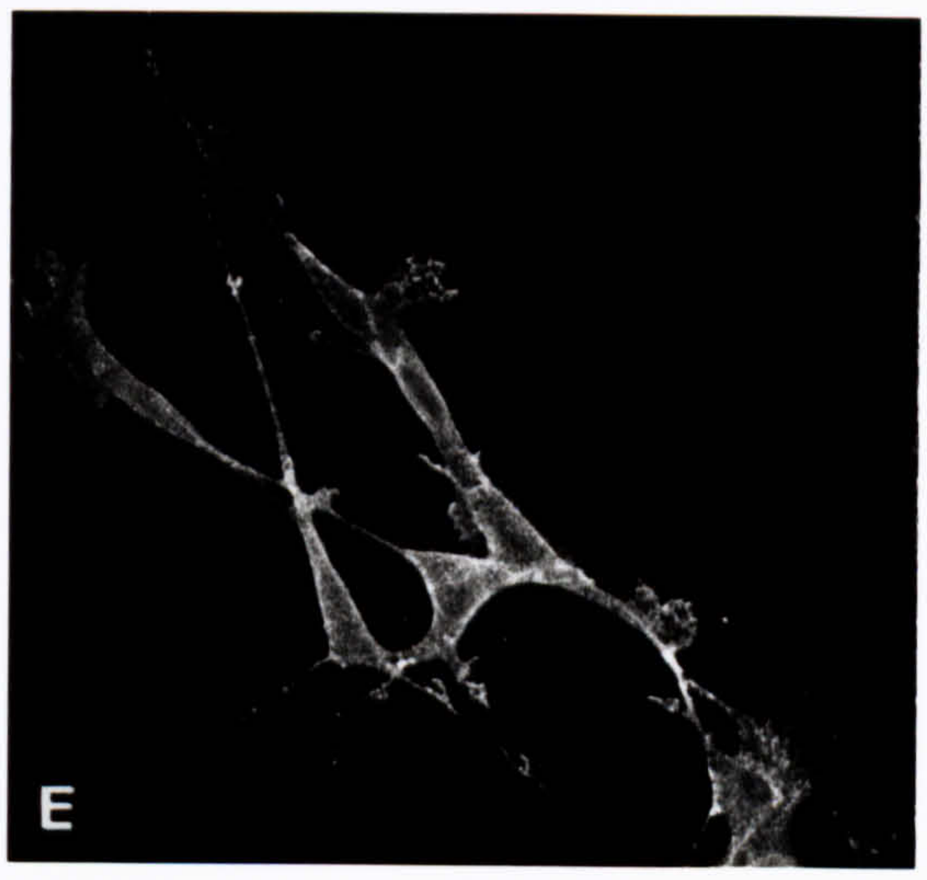
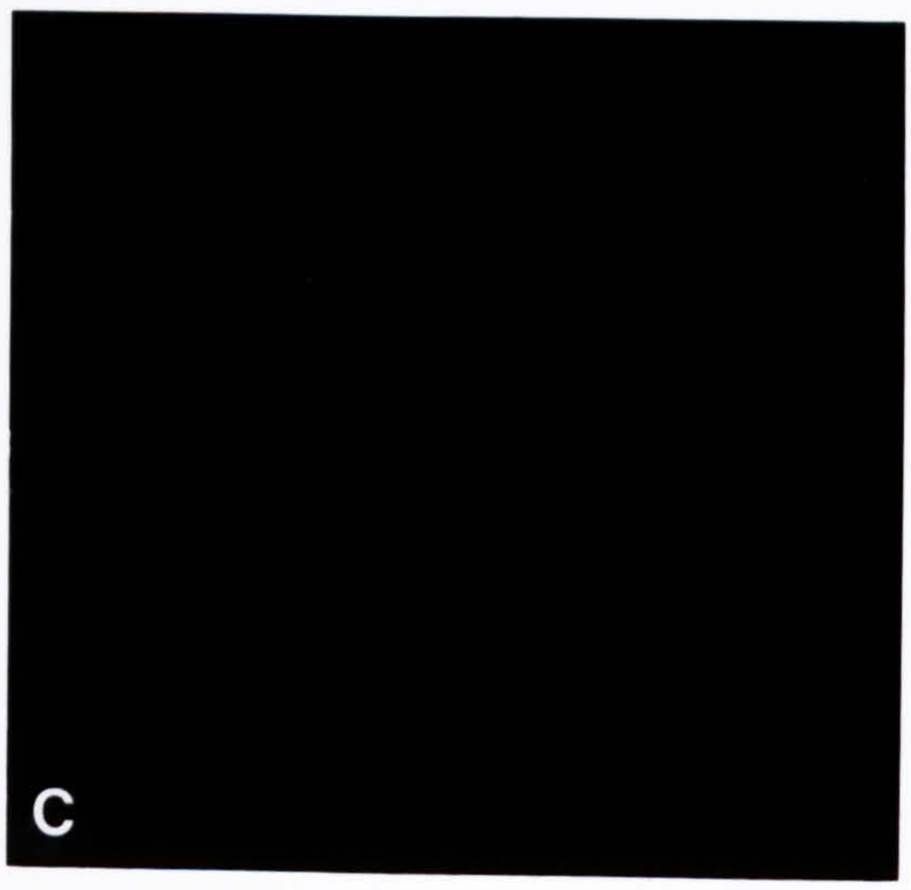

B


E


C


F

Figure 3.2 Morphology of cells isolated from E14 nerves and perinatal nerves

(A) At 3 hr post-plating, cells from E14 nerves exhibit a flattened, epithelial morphology, forming groups of cells with extensive cell-cell contacts. (C) Schwann cells from newborn nerves cultured under the same conditions exhibit an elongated bi- and tri-polar morphology. Cells cultured from E18 (B) nerves show an intermediate morphology, with some flattening of the cell body and more cell contacts than those seen in (C). (D), (E) and (F) show p75LNGFr expression on the cells shown in (A), (B) and (C) respectively. Bar = 40μm.

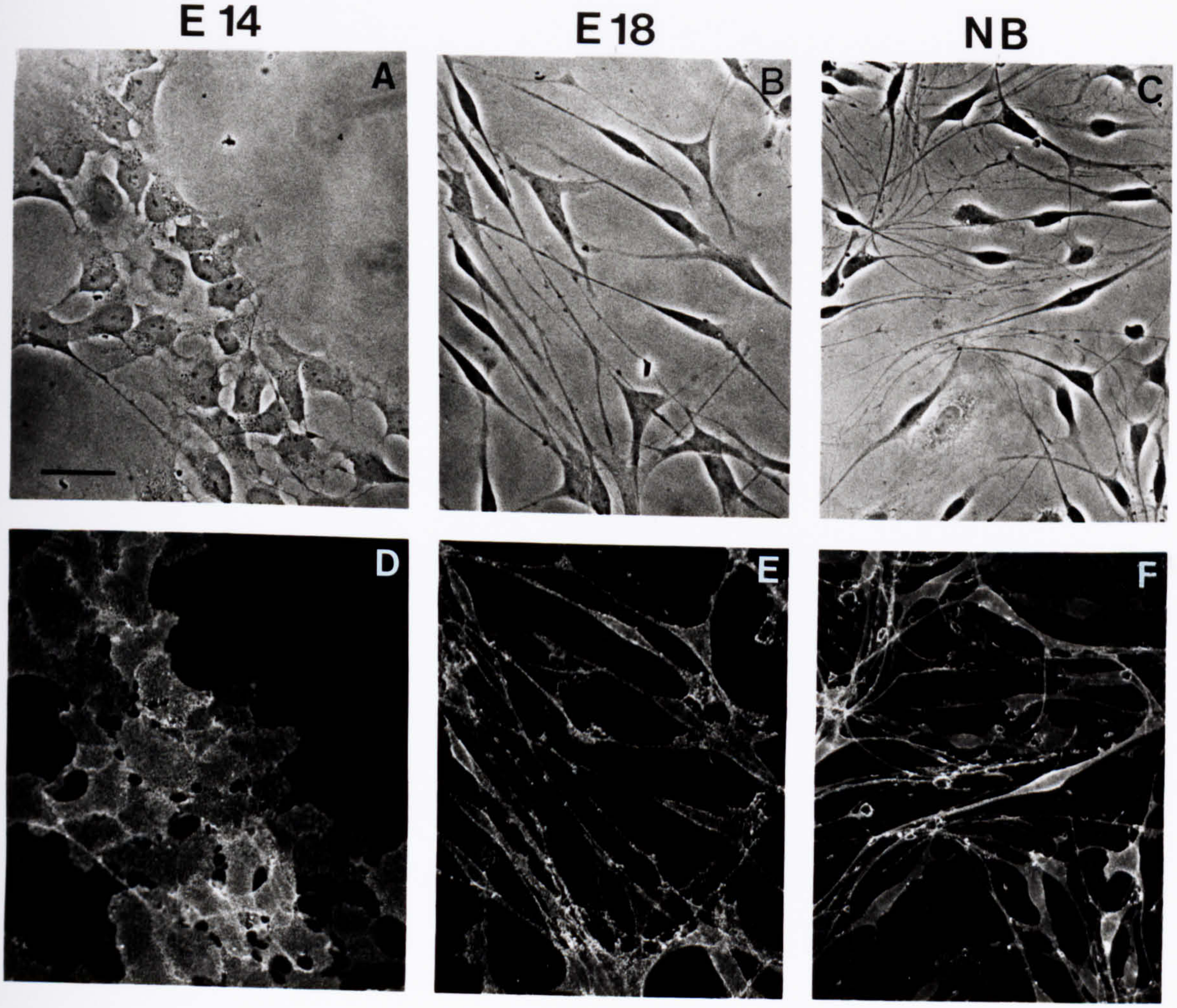

E 14
E 18
NB
A
B
C
D
E
F

Figure 3.3 The developmental appearance of S100 immunoreactivity

Cells freshly isolated from nerves of different developmental ages were double immunolabelled for p75LNGFr and S100. The change in the percentage of $p75LNGFr^{+}$ cells that express S100 immunoreactivity with increased developmental age is shown. Each point represents the average from three experiments, error bars indicate SEM, n=3.

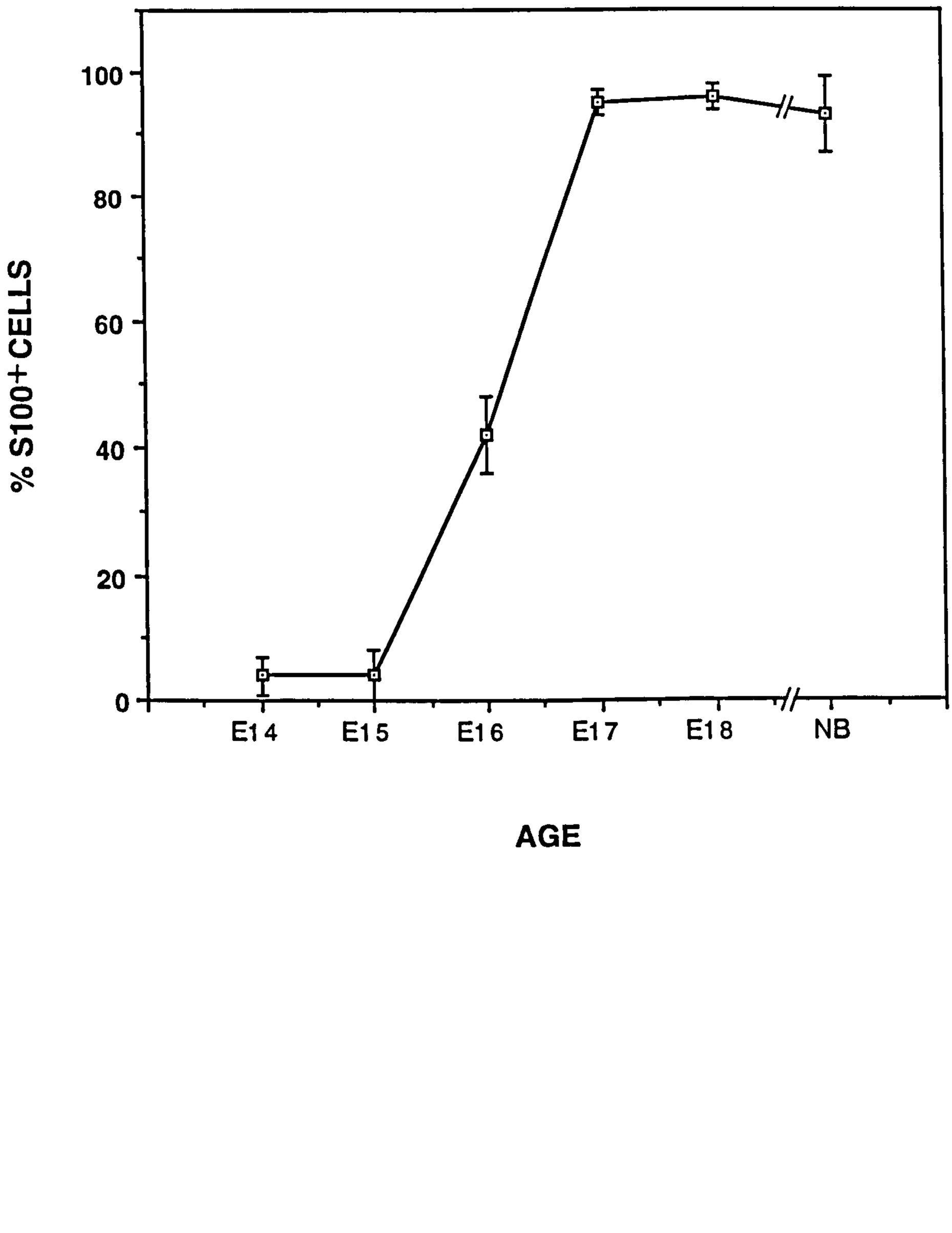

% S100+CELLS
100
80
60
40
20
0
E14
E15
E16
E17
E18
NB
AGE

Figure 3.4 Comparison of survival in defined medium between E14 and newborn cells

In a 20 hr survival assay, essentially all p75LNGFr$^+$ cells from E14 nerves die, whereas p75LNGFr$^+$ cells from newborn nerves live. The percentage survival is the number of p75LNGFr$^+$ surviving at 20 hr post-plating expressed as a percentage of flattened p75LNGFr$^+$ cells 3 hr after plating. The survival is an average from three experiments, error bars indicate SEM, n=3.

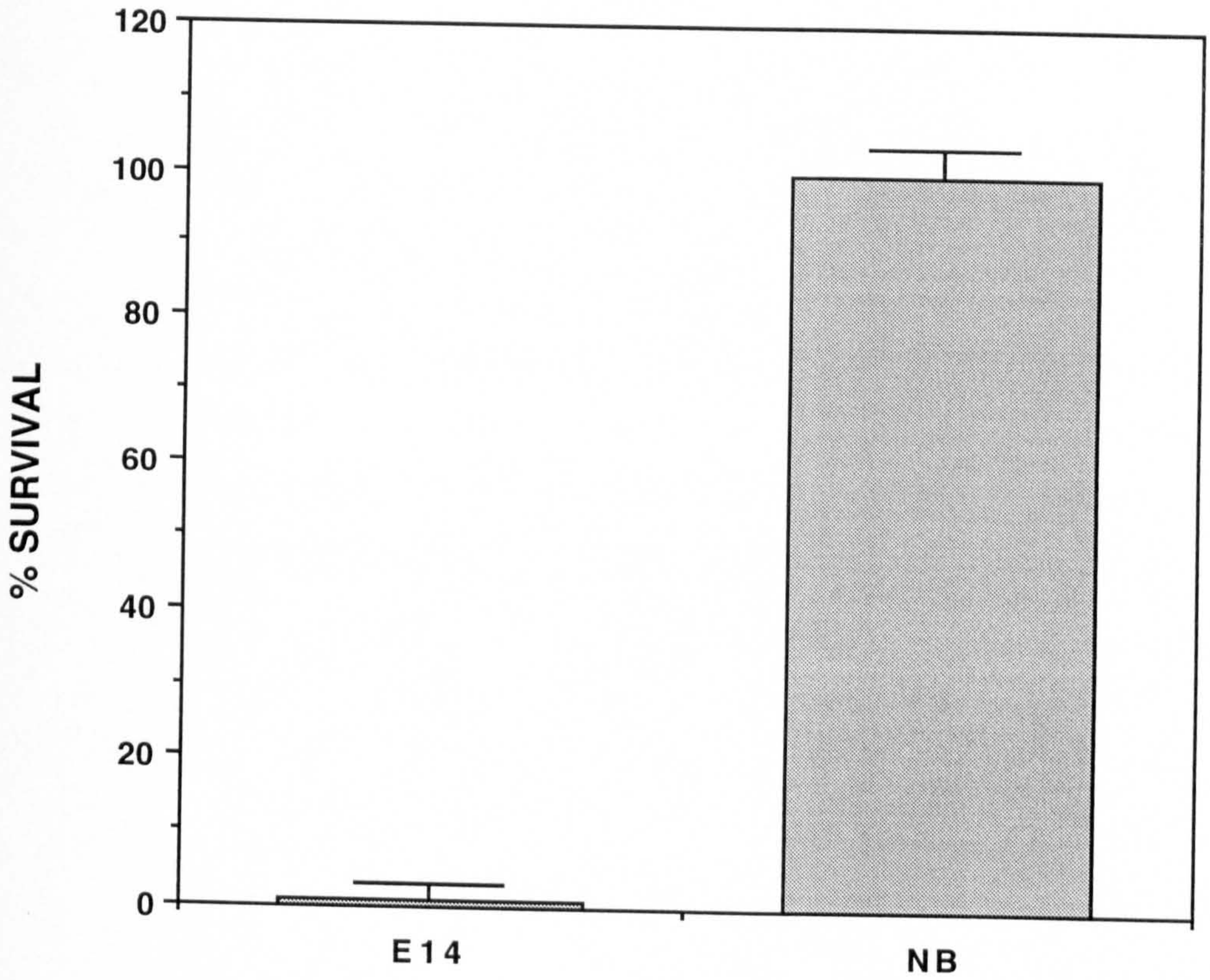
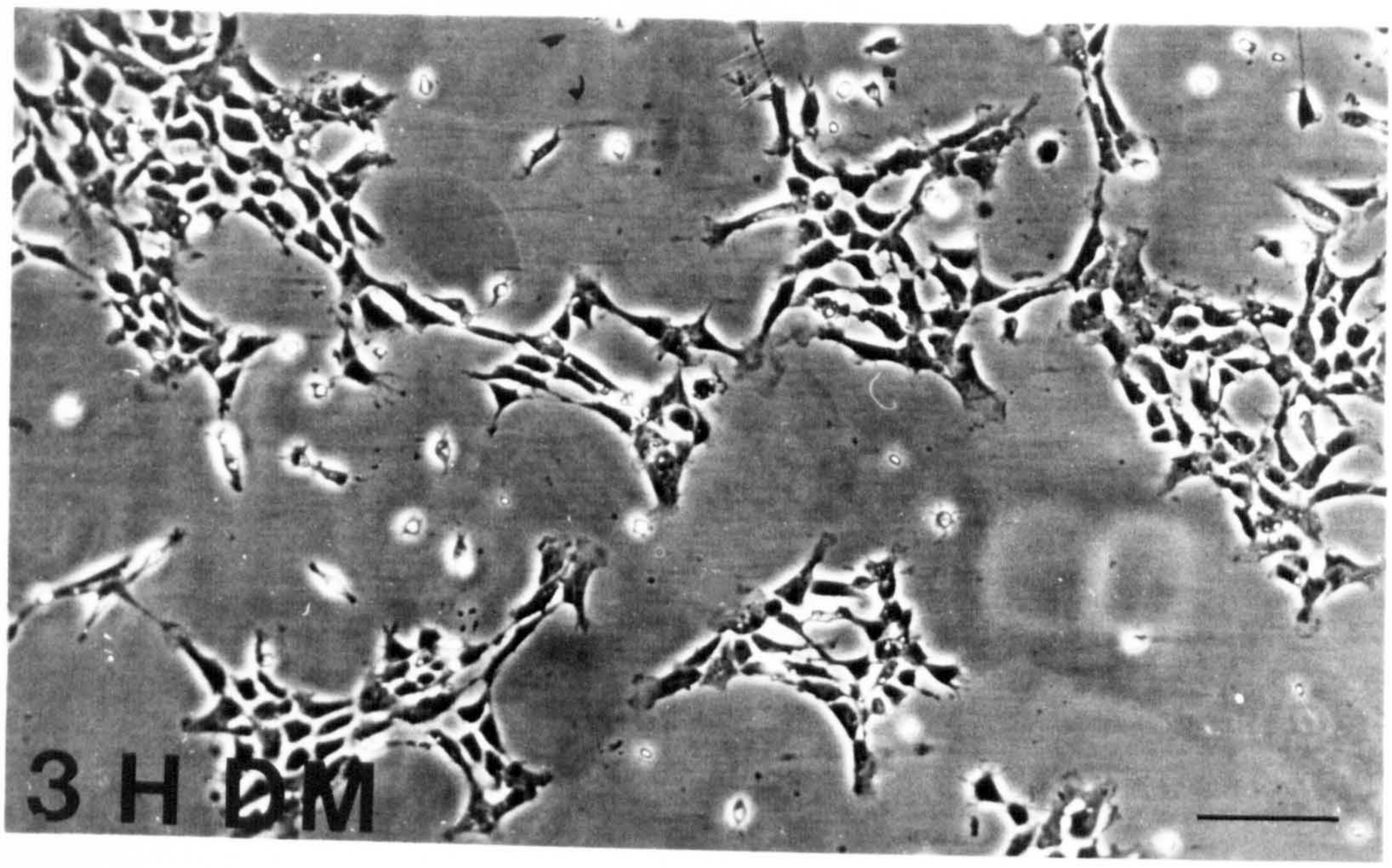

120
100
80
60
40
20
0
% SURVIVAL
E14
NB
AGE

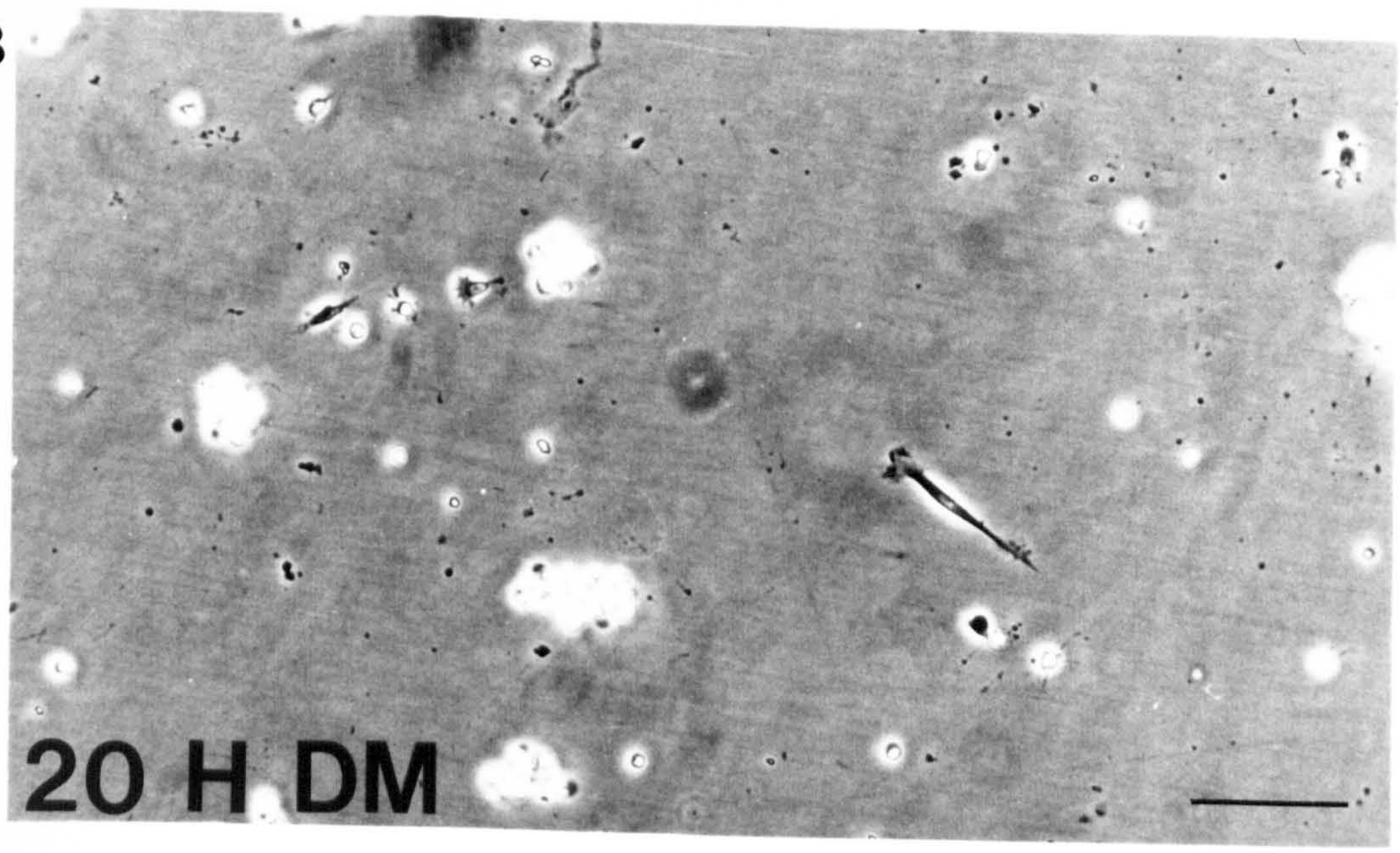
Figure 3.5 Precursors cultured under different conditions - morphology and cellular relationships

Three hours after plating in non-conditioned defined medium (A, 3 H DM), E14 precursors have flattened and formed small groups of cells. Seventeen hours later in the same medium (B, 20 H DM), the cells have died, leaving debris and rounded cells in the culture medium. If the cells are changed into NCM at 3 hr, they survive and appear 17 hr later (C, 20 H NCM) as extended, flattened cells, forming large cellular sheets. The figures show unfixed cells photographed with phase contrast optics. Bar = 50μm.

A



B



C

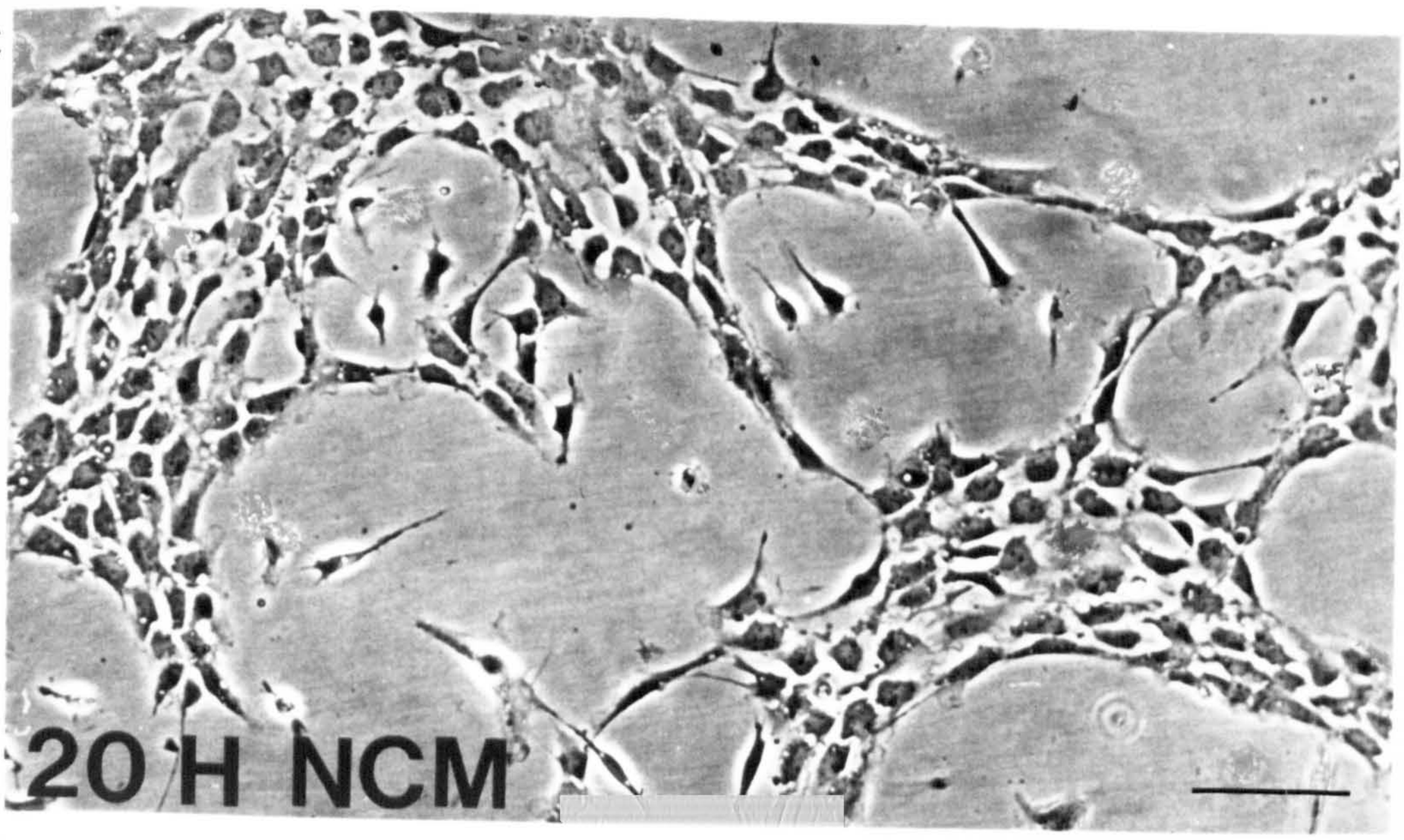


Figure 3.6 Developmental regulation of survival ability in the Schwann cell lineage

Using the 20 hr survival assay (described in legend to Fig 3.4), cells from nerves of different age rats show varied abilities to survive culture in defined medium. The ability to survive in this assay is acquired rapidly between E16 and E17. Each point represents the average from three experiments, error bars indicate SEM, n=3.

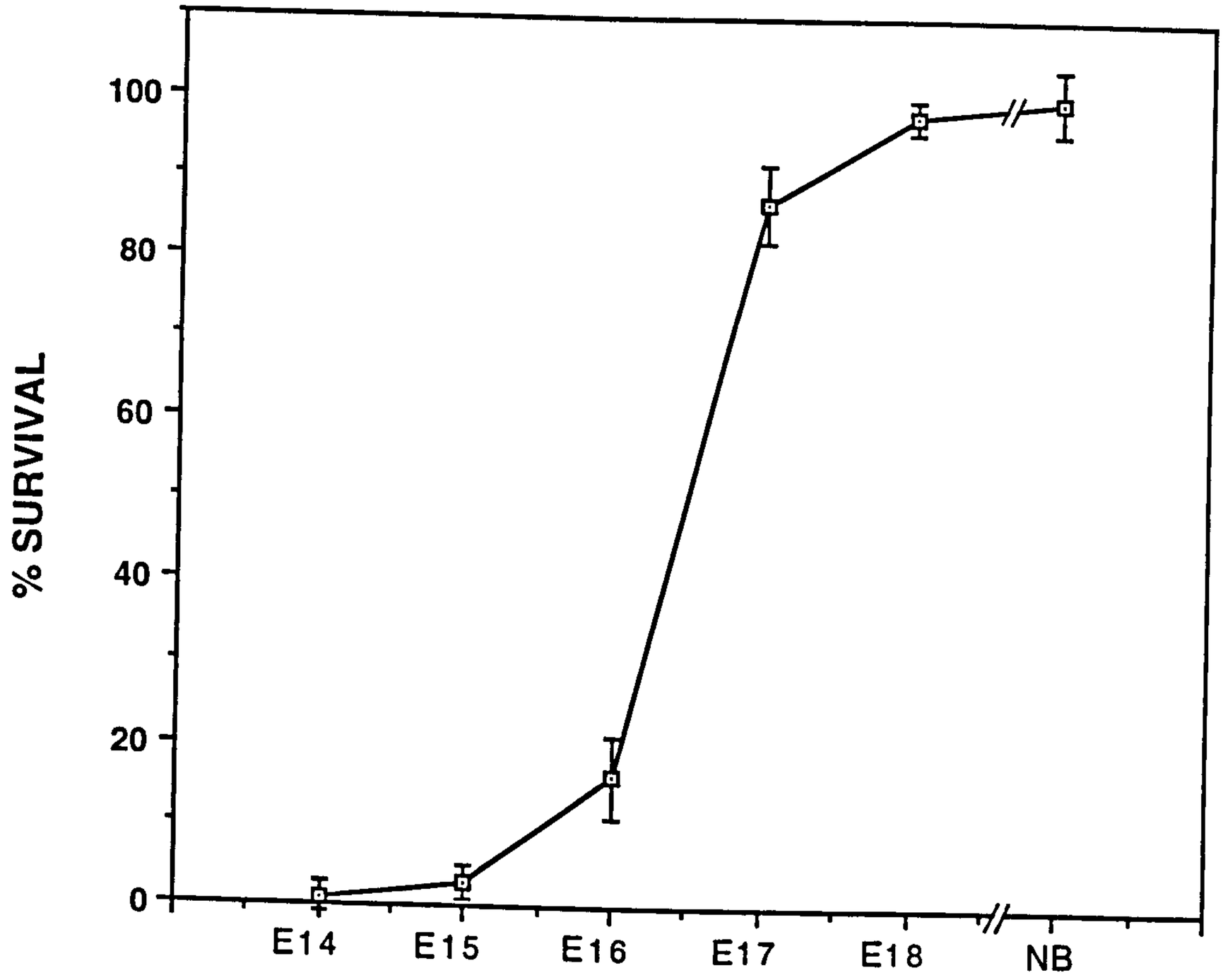

100
80
60
40
20
0
% SURVIVAL
E14
E15
E16
E17
E18
NB
AGE

Figure 3.7 The antigenic phenotype of E14 precursors

The cells were immunolabelled after culture in NCM for 18 hr with antibodies as indicated and photographed using fluorescence optics (upper panels) and phase contrast (lower panels). VIM: vimentin. Bar = 20μm.

VIM N-CAM LI A5E3 RAN-2

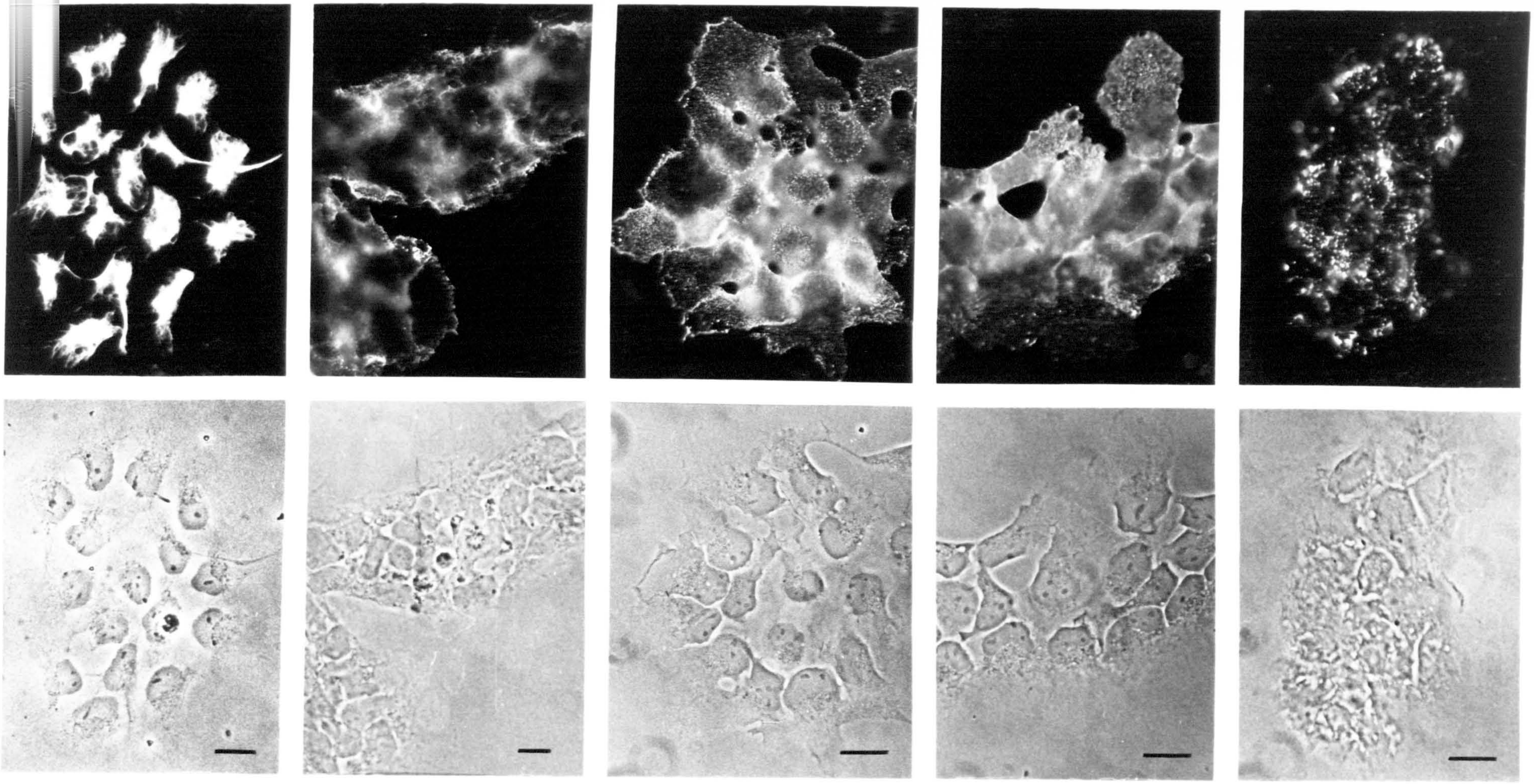
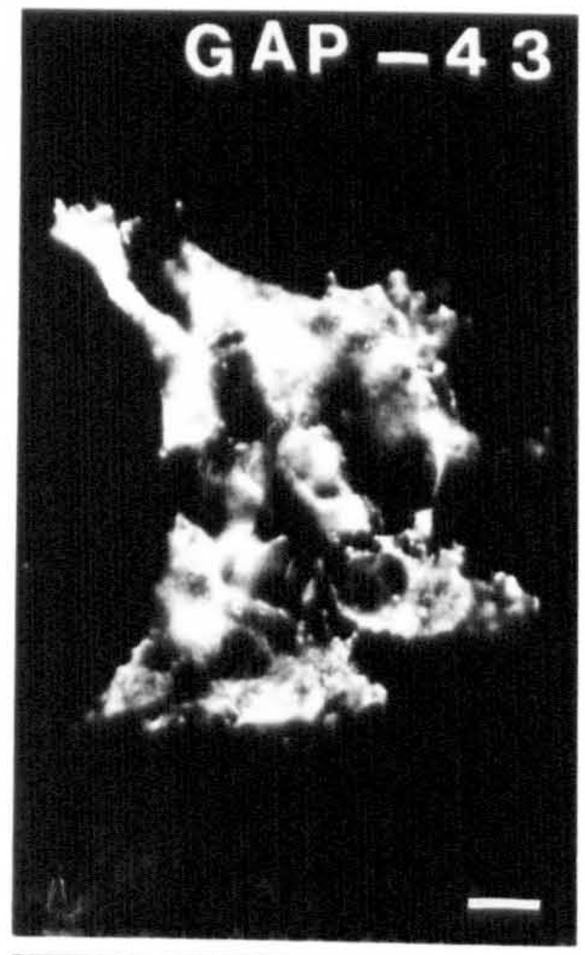
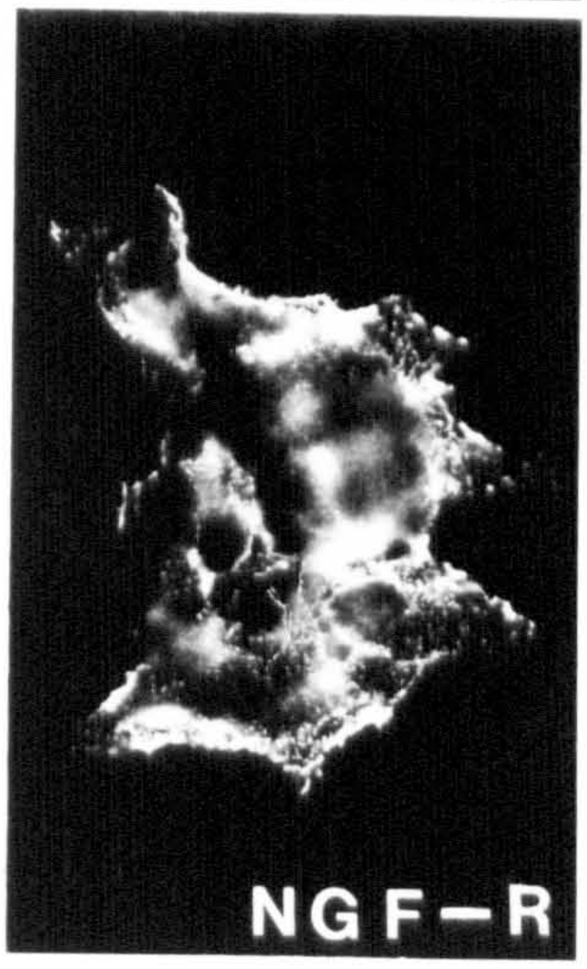


Figure 3.8 GAP-43 expression distinguishes precursors from neural crest cells

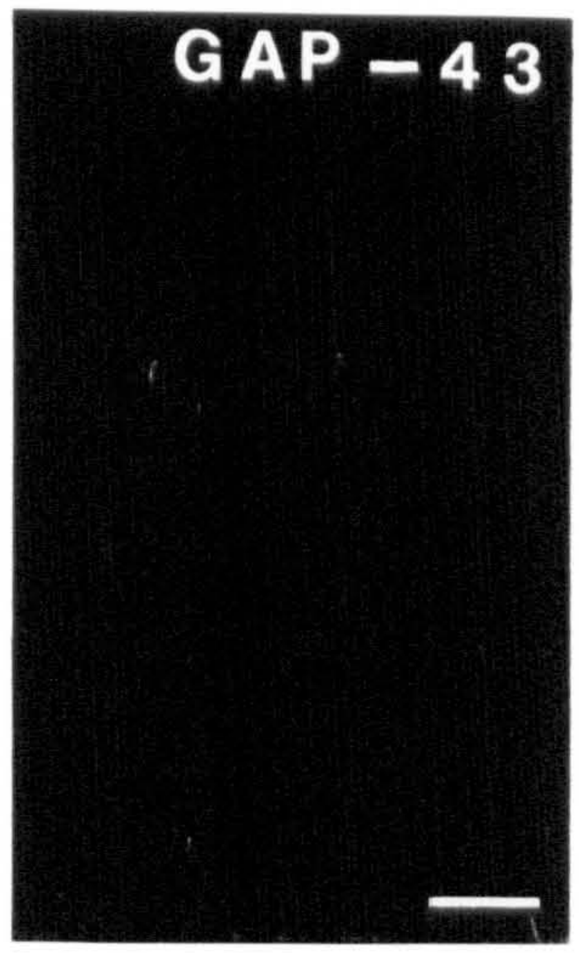
E14 precursors (E14) and neural crest (CREST) were double immunolabelled for p75LNGFr and GAP-43. The photographs show a group of 10-15 E14 cells and the edge of a flattened sheet of crest cells migrating away from the neural tube, both after 20hr in culture. The p75LNGFr labelling is similar in both cases, whereas only the E14 cells are strongly immunolabelled with GAP-43 antibodies. Exposure times for the GAP-43 photographs were identical for both E14 and crest cells. Bar = 20μm.

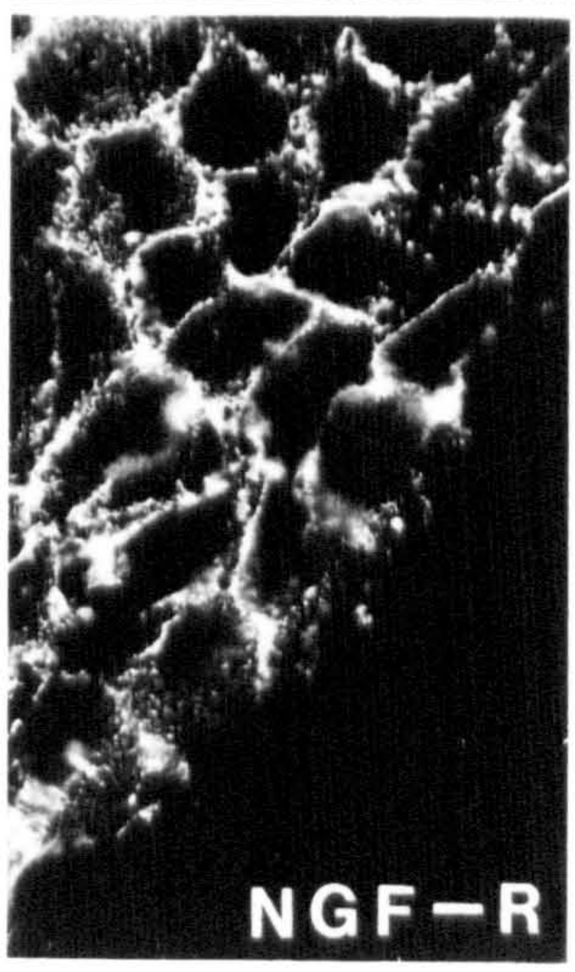
E14





CREST





DISCUSSION

Identification of the rat E14 glial cell in culture

Cells isolated from the nerve at E14 expressed the low affinity p75LNGFr, as determined by the antibodies 192-IgG and 217c. These antibodies have been shown to label rat neural crest cells and Schwann cells in culture (Smith-Thomas and Fawcett, 1989; Jessen et al., 1990; Bannerman and Pleasure, 1993). Unlike perinatal Schwann cells, however, the early glial cells did not express, or expressed at very low levels, the Ca^{2+}-binding protein S100. This protein is a marker for all perinatal and adult peripheral glia and its absence could indicate that these cells are not of the Schwann cell lineage. Indeed, the developing muscle found in the limb at this time strongly expresses p75LNGFr (Yan and Johnson, 1988; Wheeler and Bothwell, 1992). However, the distinct pavement-like morphology, combined with the expression of other markers found in peripheral nerve, for example NCAM, L1, A5E3 and nestin, indicated that the cells isolated at E14 were not mesenchymal in origin.

E14 glia can be distinguished from perinatal Schwann cells by morphology, S100 expression and survival ability

Schwann cells isolated from perinatal nerves can be cultured in defined medium where they exhibit a typical spindle-shaped morphology and can be identified by their expression of the Ca^{2+}-binding protein S100. It was surprising, therefore, to find that glial cells from E14 and E15 nerves were strikingly different, with a squamous morphology, an absence of S100 protein and a requirement for additional factors to enable the cells to survive culture in defined medium.

Morphology

Schwann cells taken from E18 and newborn (or older) nerves assumed an elongated bi- or tripolar morphology in culture, with few cell-cell contacts (Fig 3.2 B, C). Cells from E14 and E15 nerves adopted a more epithelial morphology: flattened pavements of cells were formed, with extensive cell-cell contact and few elongated processes (Fig 3.2 A). An intermediate morphology was seen at E16 and E17 (not shown), the cells having elongated processes but with more extensive cell-cell contacts than those seen at E18.

The morphology of the E14 cells was reminiscent of neural crest cells in culture, although the E14 cells appeared to have lamellar processes that were absent from the neural crest cells (compare Fig 3.2 A and 3.8) and the cell-cell contacts were not as continuous as those seen in areas of crest cell outgrowth from cultured neural tubes. As described previously, E14 cells *in vivo* ensheath large numbers of small diameter axons with fine lamellar processes extending into the developing axonal bundles (Jessen et al,. 1994), these processes appear to be retained to some degree *in vitro*.

With increased developmental age, the numbers of axons associated with each glial cell decreases (Peters and Muir, 1959; Webster et al., 1973). The change in morphology between the early glial cells and those found in the nerve from E17 onward is likely to reflect the maturation of these cells, enabling them to assume the appropriate relationships with the growing axons that will eventually lead to the formation of myelin sheaths or non-myelinated fibres.

S100 expression

When cells of different developmental ages were dissociated, plated in very short term cultures, and immunolabelled with anti-S100 antibodies, a change in S100 expression was seen with increasing developmental age. The expression of this Ca^{2+}-binding protein was first seen faintly in the nucleus at E15-E16 and appeared in the cytoplasm between E16 and 17. The level of expression, judged by the intensity of immunolabelling, increased with increasing developmental age up to birth. This appearance of S100 protein in cultured cells between E16 and E17 was also seen in sections of nerve from these ages (Jessen et al., 1994) indicating that S100 expression is developmentally regulated *in vivo*.

The appearance of S100 in peripheral glial cells during development has been documented in quail DRG (Holton and Weston, 1982a) and in chick DRG and sciatic nerve (Bhattacharyya et al., 1992). Holton and Weston (1982a) observed that glia isolated from embryonic quail DRG acquire low levels of S100 expression when cultured in the absence of neurones. This expression is upregulated in the presence of neurones and appears to require cell-cell contact. The promotion by neurones of S100 expression in early glia has also been reported in co-cultures of rat embryonic retinal neurones and neural crest cells (Smith-Thomas et al., 1990). In these co-cultures, the differentiation of neural crest cells into Schwann cells, as determined by bipolar

morphology and S100 expression, was enhanced by the presence of the neurones, both temporally and quantitatively.

Since S100 expression appears to be intimately associated with the Schwann cell precursor to Schwann cell transition, it is interesting to speculate on the role of this protein in this transition. S100 belongs to the calmodulin-S100-troponin C superfamily of proteins that contain EF-hand Ca^{2+}-binding sites (Donato, 1986; Zimmer et al., 1995). Three isoforms of S100 have been described, dimers of α or β subunits: S100ao ($\alpha\alpha$), S100a ($\alpha\beta$) and S100b ($\beta\beta$) (reviewed in Donato, 1991). Each subunit is a small acidic protein (approximately 10.5 kD) with two binding sites for calcium. The S100β subunit can also bind Zn^{2+} with high affinity, which in turn increases the S100 binding affinity for Ca^{2+} (Donato, 1991).

Originally S100 was considered to be specific to the nervous system, in particular to glial cells, and has been used extensively as a marker for glia within the PNS. More recently, however, the different isoforms have been found in many cell types, but S100b is very highly expressed in peripheral glial, and both S100a and S100b are found in glial cells of the CNS. The antibody used in these studies recognises both the α and β subunits (technical information from Dakopatts) but Schwann cells have been reported to express only β subunits (Spreca et al., 1989). Currently, there are 16 proteins identified as members of the S100 family that show amino acid sequence homology and structural similarities; the expression of these proteins is cell-type specific (reviewed in Zimmer et al., 1995). The amino acid sequences of the S100 proteins have been highly conserved between species suggesting an important biological role.

There is increasing evidence that the S100 proteins are multifunctional and that they play an important role in modulating the response of cells to extracellular stimuli. The S100 proteins are themselves modulated by calcium: binding of Ca^{2+} results in a conformational change which exposes amphipathic amino acids that are believed to bind to target proteins (reviewed in Zimmer et al., 1995). Some of the putative target proteins have been described in several cell types, but as yet there is an incomplete picture of the role of S100 in the nervous system.

An obvious function for S100 is to control intracellular Ca^{2+} levels. The presence of high levels of Ca^{2+} have been reported to induce or prevent cell death depending on

the system under study. For example, treatment of thymocytes with glucocorticoids results in Ca^{2+}-influx and apoptosis; this effect can be mimicked using the Ca^{2+}-ionophore A23187 and inhibited by removal of Ca^{2+} from the medium (Ellis et al., 1991; Compton and Cidlowski, 1992). However, an increase in intracellular Ca^{2+} as a result of membrane depolarization of cultured sympathetic neurones acts to suppress death of these cells (Edwards et al., 1991). In Chapter 5, addition of Ca^{2+}-ionophore to precursors does not prevent death, and it may be that high intracellular levels of Ca^{2+} cause death in these cells. Because of the multiple binding sites for Ca^{2+}, expression of S100 could act as a buffer of Ca^{2+}-levels within Schwann cells. However, reports from work in skeletal muscle with S100ao suggest that this form of S100 releases Ca^{2+} from sarcoplasmic reticulum (Zimmer et al., 1995) suggesting that simple control of Ca^{2+} levels is unlikely to be the prime function of S100 in Schwann cells.

S100 has been reported to act as a mitogen for C6 glioma cells and primary astrocytes (Selinfreund et al., 1991). Expression of S100 begins at a time when glial cell division starts to increase in the nerve, therefore it is possible that it is acting to control the response of the Schwann cell precursors to mitogens. It is expressed highly, however, in mature myelin-forming Schwann cells (Mata et al., 1990); these cell do not divide under normal conditions but will divide in the distal nerve after axotomy, a region where the level of expression of S100 has been reported to decrease post-lesion (De Leon et al., 1991).

There is some evidence for S100 as a neurotrophic factor since it maintains embryonic chick cortical neurones *in vitro* (Winningham-Major et al., 1989) and promotes chick motor neurone survival *in vivo* (Bhattacharyya et al., 1992). It also stimulates neurite extension from cultured embryonic chick cortical neurones (Winningham-Major et al., 1989) and sensory neurones from embryonic chick and rat DRG (Van Eldik et al., 1991). These effects depend on disulphide linked dimers of S100β and are blocked by the use of anti-S100 antisera. Treatment of the PC12 cell line with S100, however, results in an increased intracellular Ca^{2+} level and does not produce neurite extension, inducing the cells to die by apoptosis (Fano et al., 1993). Since S100b expression appears to be coincident with the transition from the survival factor-dependent Schwann cell precursor to the Schwann cell that can be maintained with out additional

growth factors, it could be postulated that S100 is a survival factor for Schwann cells. However, two observations from later work with Schwann cell precursors suggest that S100 is not an absolute requirement for growth factor-independent Schwann cell survival: i) addition of exogenous S100β does not promote the survival of precursors (data not shown) and ii) when precursors are cultured in the presence of survival-promoting growth factors that allow progression to a Schwann cell phenotype, most cells become $S100^+$ and survive culture in defined medium, but a small proportion of cells survive under these conditions and do not express S100 protein (described in Chapter 6).

S100 can act as a modulator of enzyme activity and has been shown to prevent the phosphorylation of several substrates of protein kinase C (PKC) such as GAP-43 and neurogranin (Sheu et al., 1994,1995; Lin et al., 1994). Perhaps more importantly, S100 has also been shown to affect proteins involved in the cytoskelton by inhibition of protein phosphorylation (Baudier and Cole, 1988). S100 has both inhibitory and stimulatory effects on tubulin polymerization, increasing the microtubule disassembly rate in the presence of Ca^{2+}, possibly by sequestering tubulin, and decreasing the phosphorylation of the microtubule-associated τ protein leading to an increase in tubulin polymerization (Donato, 1985; Zimmer et al., 1995).

Other cytoskeletal proteins are targets of S100: GFAP assembly into intermediate filaments is inhibited by binding of S100 to GFAP (Bianchi et al., 1993) and decreased S100b expression in C6 gliomas results in promotion of the actin filament network (although this latter action is unlikely to be a direct effect of S100 on actin since there is no apparent binding of S100 to actin; Selinfreund et al., 1990). It is possible, therefore, that in the Schwann cell lineage S100 has a role in cell morphology. Normal C6 gliomas are bipolar, stellate cells that express S100b, but if the level of S100b expression is decreased using antisense methods the cells assume a flattened fried-egg morphology (Selinfreund et al., 1990). It is intriguing in this context that the increase of expression of S100 in the Schwann cell precursors coincides with the change from a flattened squamous morphology to a bi-or tripolar one. Thus S100 maybe involved in the cytoskeletal changes associated with the precursor to Schwann cell transition, possibly by altering microfilament and intermediate filament assembly.

Most recently, MyoD, a member of the myogenic basic helix-loop-helix (bHLH) transciption factor family that activate muscle-specific genes, has been shown to contain a consensus sequence that binds calmodulin, S100a and S100b in a calcium-dependent manner (Baudier et al., 1995). The binding of calmodulin and S100a results in an inhibition of MyoD phosphorylation by PKC. Although the binding sites for calmodulin/ S100 are distinct from the DNA binding domain of MyoD, binding of these proteins results in an inhibition of MyoD DNA binding activity. This study looked mainly at the S100 protein found in high levels in developing muscle, S100a, since this is most likely to be available for interaction with the MyoD transcription factor, but it raises another possible function of S100 within glial cells, that of transcription factor regulation. As yet, however, no bHLH transcription factors have been found in perinatal Schwann cells, and transcription factors that are known to play a role in Schwann cell development, such as those of the zinc finger family (Krox 20 and Krox 24), the leucine zipper family (CREB, jun, C/EBPα) and the POU domain family (OCT 6/ SCIP/ tst-1, OCT 1) (reviewed in Stewart et al., 1996a) have not been shown to have S100 consensus binding sites. Therefore, the regulation of transcription factors by S100 in the developing Schwann cell is purely speculative.

Survival in defined medium

In these studies, the appearance of S100 expression and change in morphology closely paralled the ability of the embryonic glial cells to survive culture in defined medium. While at E14 and E15, less than 5% of the cells that attached and flattened at 3 hr survived culture in defined medium to 20 hr post-plating , this increased to 15% at E16, and to 90% of the cells at E17 (Fig 3.6). These results suggest that the embryonic glial cells that can be isolated at E14 differentiate into Schwann cells in the nerve between E15 and E17.

The ability of the E17 and older Schwann cells to survive culture in defined medium without exogenous growth factors may be due to the autocrine production of such factors by these cells. Schwann cells from perinatal and adult nerves are known to produce growth factors that can act as autocrine factors. For example they produce all three major isoforms of TGFβ (mitogenic for Schwann cells; Ridley et al., 1989; Schubert, 1992; Einheber et al. 1995; Stewart et al., 1995), and PDGF-BB (acts as a Schwann cell mitogen in the presence of elevated cAMP or TGFβ; Davis and

Stroobant, 1990; Eccleston et al., 1990, 1993). Schwann cells also produce neurotrophic factors namely NGF, BDNF, NT-3, NT-4/5, CNTF, LIF and glial derived neurotrophic factor (GDNF) (reviewed in Scherer and Salzer, 1996) and possess receptors for some of these factors. The low affinity p75LNGFr is expressed in undifferentiated and non-myelin-forming Schwann cells and NGF is reported to increase the level of L1 expression on cultured mouse Schwann cells (Seilheimer and Schachner, 1988). Schwann cells also express mRNA of full length *trk*C, and the truncated forms of *trk*B and *trk*C lacking tyrosine kinase domains (Offenhäuser et al., 1995). It is possible that the lack of survival ability by early embryonic glial cells is due to lower levels of autocrine growth factor(s) being produced by these cells.

Plating the E14 cells at high density did not increase the proportion of cells surviving at 20 hr (Table 3.1). This may indicate that the cells cannot produce the growth factor(s) that promotes their survival at E17, or that they lack the receptors for the factor(s), or both of these. In earlier studies (Jessen et al., 1994), medium conditioned by neonatal Schwann cells could not rescue the E14 cells, suggesting that they lack the receptors for the survival factors that might be produced by older Schwann cells. Moreover, the exogenous addition of growth factors known to be produced by Schwann cells, namely NGF, BDNF, NT3, NT4, TGFβ and PDGF-AA and -BB, to cultures of E14 cells did not promote survival. These results will be described in detail in Chapter 5. These results suggest that the factors necessary for E14 cell survival must be produced by other cells in the early nerve or the surrounding environment.

The increased survival in defined medium seen at E17 could be due to proliferation of a subpopulation of cells rather than increased survival. When E17 and newborn cells were pulsed with BrdU for the last 1.5 hr of the 20 hr assay, it was found that less than 1% of cells incorporated the label, indicating they were undergoing division. These levels of division are too low to account for the numbers of cells seen at 20 hr.

Neurone-conditioned medium promotes survival of E14 glial cells

As described earlier, at all stages of nerve development the glial cells are seen in close association with the growing axons (Peters and Muir, 1959; Loring and Erickson, 1987; Dahm and Landmesser, 1988; Carpenter and Hollyday, 1992a, 1992b; Jessen et al., 1994; Y. Hashimoto and K. R. Jessen, personal communication). This close

association with neurones is known to directly influence Schwann cell gene and protein expression (reviewed in Mirsky and Jessen, 1990; Gould et al., 1992; Scherer and Salzer, 1996), upregulating expression of proteins such as S100 (Holton and Weston, 1982b), galactocerebroside (Jessen et al., 1987a), 04 (Mirsky, et al., 1990) and, in myelin-forming Schwann cells, the myelin proteins including P_0, MBP, MAG and PLP (reviewed in Gould et al., 1992; Lemke and Chao, 1988). It has also been reported that diffusible molecules from neurones can influence Schwann cell genes, downregulating p75LNGFr and inducing SCIP and P_0 in cultured adult rat Schwann cells (Bolin and Shooter, 1993). Axons are, therefore, a potential source of survival factor(s) for embryonic glia.

When defined medium was conditioned by purified DRG sensory neurones, either in the presence or absence of NGF, it promoted the survival of E14 glial cells. At 20 hr, in NCM, 80% of the E14 cells were still alive (Figure 3.5 C).

Purified axonal membrane has been shown to stimulate proliferation in Schwann cells from neonatal rats (Salzer et al., 1980a,b; Sobue and Pleasure, 1985). It was possible, therefore, that the conditioned medium was having a mitogenic effect on a small proportion of the cells and not acting as a survival factor. Pulsing the cultures with BrdU for the last 1.5 hr of culture, however, revealed that, in NCM, the level of cell division was low at less than 1%, and could not account for the numbers of cells surviving.

It is possible that the survival factor(s) found in the NCM are produced by other cells in the neuronal cultures, these are fibroblasts, satellite cells and Schwann cells. However, the levels of E14 survival seen in conditioned media from different cultures of purified DRG neurones were directly related to the purity of the neuronal cultures: the best survival was seen in medium from the purest cultures of neurones (not shown), indicating that the source of survival factor(s) was unlikely to be the contaminating cells. This has since been confirmed in similar survival experiments using immunopanned DRG neurones to condition the medium (Dong et al., 1995). Since the survival ability of the medium decreased with increasing numbers of non-neuronal cells, it is possible that these contaminating cells were either degrading the survival factors produced by the neurones, or selectively binding them and removing

them from the conditioned medium. These results indicate that neurones are likely to be the source of survival factors for early embryonic glial cells.

The molecular phenotype of the E14 glial cell

Dissociated cells cultured from E14 nerves for either 3 hr (in defined medium) or for 20 hr (in NCM) were probed with a panel of antibodies that have been shown to label Schwann cells at later stages of development (reviewed in Mirsky and Jessen, 1990; Jessen et al., 1990; Mirsky et al., 1990). The labelling after the different times in culture was the same, indicating that the short time in culture did not alter the molecular phenotype of the cells.

Table 3.2 describes the phenotype of the E14 cells compared to similar cultures of rat neural crest cells. These results are compared to those seen in myelin- and non-myelin-forming Schwann cells in Table 3.3.

These early cells express three proteins that label both myelin- and non-myelin-forming Schwann cells, the extracellular matrix molecule laminin (although this expression was weak) and the intermediate filament proteins vimentin and nestin. As described earlier, they fail to express S100, found in all Schwann cells. The majority of the early glial cells express markers that are normally associated with non-myelin-forming Schwann cells, namely p75LNGFr, the cell adhesion molecules NCAM and L1, and the cell surface molecules of unknown function A5E3 and Ran 2. However, unlike the non-myelin-forming Schwann cells, these cells do not express the intermediate filament protein GFAP.

Late embryonic Schwann cells express the lipids galactocerebroside and O4 (Mirsky et al., 1980, 1990) but these are absent from the glial cells found at E14. The appearance of these lipids preceeds the expression of high levels of the myelin protein P_0 and expression of MBP in myelin-forming cells, normally seen in the nerve from birth onwards (reviewed in Gould et al., 1992). In accordance with the absence of lipids, the myelin protein MBP and high levels of P_0 are also not expressed in the Schwann cell precursors. Thus these cells more closely resemble the phenotype of the non-myelin-forming Schwann cells.

The low level of P_0 expression specifically in the precursors is in agreement with observations made in the chick, where expression of this protein can be detected in a

sub-population of neural crest cells (Bhattacharrya et al., 1991). Studies of the mRNA expression in the rat have confirmed that, as in chick, P_0 mRNA can be detected in a sub-population of migrating neural crest cells (Lee, Brennan et al., in preparation). Since this protein exhibits homotypic binding, it may be involved in adhesion of the neural crest cells to one another during migration. It is not known whether expression of the protein indicates commitment to a glial lineage.

The phenotype of these cells suggests that these are early cells of the Schwann cell lineage and not cells of mesenchymal origin despite their lack of S100 expression. The expression of L1 is not detected on cells isolated from the mesenchyme (not shown) and appears to be very specific for the Schwann cell precursors in tissue derived from the embryonic limb. In later studies such as those described in Chapter 6, L1 has been used as a specific marker for precursors: approximately 3-5% of cells labelled by p75LNGFr are negative for L1, suggesting that some of the p75LNGFr$^+$ cells are not of the Schwann cell lineage (Z. Dong, personal communication).

E14 cells are not migrating neural crest cells

Neural crest cells are highly motile cells, migrating long distances within the developing embryo and forming most cell types of the peripheral nervous system. Much evidence points to these cells giving rise to Schwann cells, although there is no *in vivo* evidence for this in the rat. It is possible that the cells seen associated with the developing nerve at E14 are migrating crest cells, capable of differentiating into several cell types, with no commitment to the Schwann cell lineage at this stage. Although there is no evidence from this work that the cells of the early nerves cannot assume other developmental fates, this study shows that there are differences between neural crest cells and E14 glial cells cultured under the same conditions. Thus the cells have advanced from the neural crest stage of development.

In culture, rat neural crest cells have a subtly different morphology from the E14 cells, with fewer lamella sheets and more intimate cell-cell contacts (Fig 3.8). However, they express several antigens that are found on the E14 glial cells, namely p75LNGFr, N-CAM, L1, A5E3 and Ran-2. The major antigenic difference that can be detected between these cells is the expression of the membrane-associated phosphoprotein GAP-43: this antigen is very weakly expressed or entirely absent from neural crest cells but is highly expressed in cells from E14 nerves. Although not quantitatively

measured, the level of immuofluorescence seen in the E14 cells is equivalent to the level observed in growing neurites of cultured neurones (not shown).

GAP-43 was first identified as a membrane phosphoprotein in neurones (reviewed in Skene, 1989) and is associated with the membrane of growth cones in development or regeneration (Skene et al., 1986). This protein is likely to have a role in membrane-cytoskeleton interactions, particularly where the membrane is undergoing changes or process formation (Zuber et al., 1989; Meiri and Gordon-Weeks, 1990). It is also expressed in non-myelin-forming Schwann cells, but not myelin-forming cells in the normal nerve (Curtis et al., 1992). Unmyelinated axons undergo changes in diameter associated with transport of membranous organelles (Greenberg et al., 1990) and it has been postulated that non-myelin-forming Schwann cells need to be able to alter cell shape to accommodate these alterations in axonal caliber (Curtis et al., 1992).

The presence of GAP-43 in Schwann cell precursors possibly relates to process formation, since these cells elaborate lamellar sheets and tapering processes in culture and extend fine processes into the bundles of axons in the E14 nerve (Jessen et al., 1994). They must be able to undergo extensive cytoplasmic changes at this time, accompanying the growing axons as they extend into the surrounding tissue. Neural crest cells, however, show little process formation and migrate rapidly within extracellular matrix, not forming close associations with surrounding tissue. The absence of GAP-43 within these cells may reflect the lack of cell membrane extension.

A further difference between neural crest cells and E14 precursors was the ability of IGF-1 and either NDF or ET-1 to promote long-term survival of these cells in the absence of the neural tube. In these studies, bFGF in the presence of IGF-1 acted as a short-term survival factor both for neural crest cells and for precursors (see Chapter 5); this effect of bFGF with high levels of insulin on rat neural crest cells has been reported previously (Bannerman and Pleasure, 1993). The lack of effect of NDF and ET-1 on neural crest cells may indicate that a complex combination of factors is required for neural crest survival, since crest cells express receptors for NDF (Marchionni et al., 1993) and can respond to GGF, a member of the NDF family (Shah et al., 1994; Verdi et al., 1996; described in detail in Chapter 6). Trunk neural crest cells are also influenced by ETs (described in detail in Chapter 6). These results

suggest that the maturation of neural crest cells to the glial cells found in E14 nerve involves changes in survival requirements.

Thus E14 glial cells are not migrating neural crest cells, although they share similarities such as a flattened morphology *in vitro*, an inability to survive in defined medium and high motility.

The results presented here show that the glial cells that can be isolated from the embryonic nerve at E14, the Schwann cell precursors, are intermediates in the Schwann cell lineage, lying between the neural crest cells and perinatal Schwann cells. Transition to a perinatal phenotype is rapid and involve changes in morphology, molecular phenotype and dependence on survival factors. This developmental step is tightly controlled and takes place between E15 and E17.

The following chapter will explore the cellular events that occur when E14 precursors are removed from neuronal contact and cultured without exogenous growth factors

CHAPTER 4

SCHWANN CELL PRECURSORS DIE BY APOPTOSIS IN THE ABSENCE OF GROWTH FACTORS

INTRODUCTION

Cell death has long been known to play an important role in normal animal development (Glücksmann, 1951; Snow, 1987; Ellis et al., 1991). Cell death is also vital in homeostasis; cell renewal must carefully balance cell proliferation and death. It is during embryogenesis, however, that normal cell death is involved in generating the final body plan of the animal and is responsible for regulating both the cell numbers and phenotypes within a tissue (Snow, 1987). Most tissues have been found to undergo periods of cell death connected with morphological changes or elimination of unwanted cells (Glücksmann, 1951). For example, neural tube closure requires cell death as does palatine fusion in mammals, formation of digits from the limb primordia and loss of transitory structures such as the tadpole tail (Snow, 1987). In the immune system, normal cell death is responsible for the elimination of self-reactive T cells (Cohen, 1991). Cell death is also an essential component in generation of both central and peripheral nervous systems where excess cells and those forming inappropriate connections must be eliminated.

The morphology of apoptosis

Normal cell death is referred to as apoptosis or programmed cell death (Wyllie et al., 1980; Kerr et al., 1987). The process of apoptosis follows similar patterns in many different cell types with these patterns appearing to be conserved between species (Ellis et al., 1991). It was thought to differ from necrotic death in morphological detail and in the response of the surrounding cells to the products of cell death (Wyllie et al., 1980; Kerr et al., 1987). In apoptosis, the cell cytoplasm and nucleus condense and the cell is fragmented into many small discrete vesicles which are rapidly phagocytosed by surrounding cells, preventing any inflammatory reaction and maintaining the integrity of the surrounding tissue (Wyllie et al., 1980). Necrotic death, however, results in swelling of the endoplasmic reticulum and mitochondria, followed by rupture of the plasma and organelle membranes. The resulting release of cell contents produces in an inflammatory reponse within the surrounding tissue (Wyllie et al., 1980). The two modes of cell death may activate some of the same pathways: recently, DNA fragmentation that has been thought to be a characteristic of

apoptosis has been detected within cells exhibiting morphological changes associated with necrosis (reviewed in Bortner et al., 1995). In addition, not all types of cell undergoing apoptosis will exhibit all of the changes classically associated with this form of cell death (Bortner et al., 1995).

An early event in apoptosis is the condensation of chromatin to the outside edges of the nucleus which may assume an irregular outline (Wyllie et al., 1980). This condensation is thought to involve a domain nuclease that cleaves the DNA into 50-300 kb fragments (Bortner et al., 1995; Earnshaw, 1995a). The nuclear membrane remains intact but the nuclear pores are redistributed to accumulate between the areas of condensed chromatin as a result of nuclear lamina disassembly by proteolysis (Earnshaw, 1995b). The nucleus may then fragment into small, highly condensed (pyknotic) chromatin bodies, only some of which are bounded by nuclear membrane (Wyllie et al., 1980; Kerr et al., 1987).

Cytoplasmic condensation accompanies the nuclear changes, associated with varying degrees of membrane blebbing and vacuole formation (Kerr et al., 1987). Organelles remain intact throughout the process of apoptosis (Wyllie et al., 1980). Eventually the cytoplasm separates into many apoptotic bodies, some containing the condensed chromatin, all of which are sealed by a plasma membrane (Wyllie et al., 1980; Kerr et al., 1987). Changes in the cell membrane must occur that allow these apoptotic bodies to be rapidly phagocytosed and degraded by neighbouring cells (Cohen, 1991). The time taken to completely eliminate a cell by apoptosis varies according to cell type (Earnshaw, 1995b) but can be extremely rapid, a matter of a few hours for cells of the immune system (Cohen and Duke, 1984).

Control of apoptosis

Apoptosis is an active process requiring the action of nucleases and proteases (reviewed in Earnshaw, 1995a). Various conditions have been identified as a trigger for apoptosis, with different cell types being sensitive to different conditions. The most common signals *in vivo* appear to be variation in hormonal or growth factor levels, for example immature thymocytes die when exposed to glucocorticoids (Cohen, 1991), sympathetic neurones will die *in vitro* if deprived of NGF (Martin et al., 1988). Apoptosis is also induced in many cell types by exposure to radiation. Recently two factors have been identified that actively induce cell death when bound to their

appropriate receptors: tumour necrosis factor (TNF) and Fas ligand (FasL) (reviewed in Cleveland and Ihle, 1995). The latter is thought to be responsible for the elimination of self-reactive T cells and to play a role in T cell-mediated cytotoxicity.

Some of the genes involved in the apoptotic process were originally determined in mutations of C. elegans (reviewed in Ellis et al., 1991; Ellis, 1992; Freeman et al., 1993). Two genes are required to be active for death to occur: *ced 3* and *ced 4* (Ellis and Horvitz, 1986; Yuan and Horvitz, 1990). Mutation of either gene results in the survival of cells that normally die. The action of these genes appears to be blocked by the product of the *ced-9* gene. This gene acts to prevent death: mutations that activate *ced-9* prevent cell death and mutations that delete the gene are fatal, resulting in loss of cells that would normally survive. These studies indicate that cell death is suppressed in all cells that normally survive (Ellis et al., 1991). Determination of which cell should undergo cell death seems to be controlled by separate genes according to cell type in steps that occur before the activation of *ced-3* and *ced-4* (Ellis et al., 1991). At least 7 different genes are responsible for engulfment of the dead cells.

Cell death genes in mammalian systems

Cell death genes have been sought in vertebrates and several homologues of the C. elegans genes have been identified. A homologue of the death repressor *ced-9* is the mammalian gene *bcl-2*. This was originally isolated from a human B cell lymphoma (Tsujimoto et al., 1985) and encodes a membrane protein that appears to be associated with the inner mitochondrial membrane, nuclear envelope and parts of the endoplasmic reticulum (Hockenbery, et al., 1990; reviewed in Wang et al., 1995). Introduction of the human *bcl-2* gene reduces the number of cell deaths when introduced into C. elegans (Vaux et al., 1992). Overexpression of the gene protects various mammalian cells from cell death, for example PC12 cells transfected with *bcl-2* can survive in the absence of NGF (Mah et al., 1993), and in transgenic mice the numbers of neurones dying during the period of normal cell death is reduced (Martinou et al., 1994). There are some situations, such as neurones after CNTF withdrawal, where *bcl-2* does not seem to prevent apoptosis (Allsopp et al., 1993). Several homologues of *bcl-2* have now been identified including *bcl-x* (Boise et al., 1993), *bax* (Oltvai et al., 1993), *bad* (Yang et al., 1995) and *bak* (Chittenden et al.,

1995; Farrow et al., 1995; Klefer et al., 1995). The Bax protein has been shown to dimerise with Bcl-2 and both proteins can form homodimers (Oltvai et al., 1993; Middleton et al., 1996). The formation of heterodimers of Bax with Bcl-2 reduces the ability of the latter protein to prevent apoptosis and thus the two proteins are thought to act as regulators of the cell death process, with the outcome dependent on the ratio of the two proteins (Oltvai et al., 1993; Middleton et al., 1996). Overexpression of Bax in various neuronal types can prevent some neuronal death after growth factor withdrawal but actively suppresses survival of these neurones in the presence of growth factors, implying a complex interaction between these proteins and the process of cell survival (Middleton et al., 1996).

The product of *ced-3* has been found to be 28% homologous in DNA sequence to the mammalian cysteine protease interleukin-1β-converting enzyme (ICE) (Yuan et al., 1993). This enzyme cleaves the precursor of interleukin-1β (IL-1β) to give the mature cytokine (reviewed in Kumar, 1995). It was thought that ICE might be an essential protein for apoptosis, but transgenic mice deficient in ICE still exhibit normal cell death (Li et al., 1995). A family of cysteine proteases related to ICE have been found that include *Nedd2/ Ich-1* (Wang et al., 1994; Kumar, 1995), and CPP32 (Lazebnik et al., 1994; Nicholson et al., 1995). All of these proteases are homologous to *ced-3* and all have been reported to induce apoptosis when overexpressed in various cell types (reviewed by Kumar, 1995; Fraser and Evan, 1996; Wang et al., 1994; Nicholson et al., 1995). Although the members of this protease family share an unusual substrate specificity, cleaving peptides at an Asp-X bond (where X is any amino acid), each protease appears to be active on a specific substrate (Kumar, 1995). One of the first events detectable in cell-free apoptosis systems is the cleavage of the enzyme poly (ADP-ribose) polymerase (PARP) responsible for DNA repair; PARP has been shown to be a substrate for CPP32 (also known as prICE and apopain) (Lazebnik et al., 1994; Nicholson et al.,1995). This is the first description of an activity for a *ced-3*-like gene, and it is possible that different genes in the protease family have roles in degrading different substrates or act in different cell types.

The Drosophila gene *reaper* appears to control apoptosis; its deletion blocks almost all death in embryos (White et al., 1994). Irradiation-induced apoptosis is also blocked by deletion of this gene and it is possible that several pathways to apoptosis converge

at this point (White et al., 1994; Steller, 1995). The action of *reaper* is not known but it has been suggested cell death effectors, present in most or all cells, are activated by *reaper* (White et al., 1994), or that it inhibits negative regulators of apoptosis such as *bcl-2*. So far, there are no homologues of this gene in the mammalian system.

Apoptosis in the mammalian nervous system

Extensive cell death of neurones within the developing central and peripheral nervous systems has been well documented (reviewed by Oppenheim, 1991; Hamburger and Levi-Montalcini, 1949; Cowan et al., 1984). In the developing PNS, it is known that neurones undergo a period of cell death, thought to be associated with limiting amount of growth factors, neurotrophins, essential for neuronal survival (Davies, 1988; Barde, 1989). The restriction of neuronal numbers by such means is thought to eliminate defective cells and aberrant connections between neurones and their targets, and to adjust the size of the neuronal population to that appropriate for the organism (Oppenheim, 1991). Neurones can be cultured *in vitro* in the presence of appropriate neurotrophins but undergo apoptosis if deprived of these growth factors (Davies, 1988; Barde, 1989).

Cell death by apoptosis during development has also been reported in glia of the CNS *in vivo* (Pannese and Ferrannini, 1967; Knapp et al., 1986; Barres et al., 1992) and as many as half of all oligodendrocytes within the optic nerve are thought to die during development (Barres et al., 1992). Barres et al. (1992) also showed that oligodendrocytes will undergo apoptosis if deprived of growth factors in culture. Recently, glial cell death during normal development of the PNS has been studied in the ventral roots of the embryonic chick (Ciutat et al., 1996). Cells identified as Schwann cell precursors by use of a monoclonal antibody that recognises chick P_0 (Bhattacharyya, et al., 1991) have been observed to die, in two waves during normal development: the first wave is early, peaking at E5-6; the second wave peaks at E8.5, and is thought to relate to the period of normal motor neurone death in the chick (Ciutat et al., 1996).

The genes responsible for the death or survival of cells within the nervous system have yet to be fully elucidated. The protein bcl-2 has been found extensively in the nervous system during development, and continues to be expressed in the adult (Merry et al., 1994). Examination of peripheral ganglia with anti-bcl-2 antibodies show expression

of this protein in both neurones and glia (Merry et al., 1994). As described above, *bcl-2* can protect neurones from apoptosis both *in vivo* (Martinou et al., 1994) and *in vitro* (Allsopp et al., 1993; Mah et al., 1993).

Homologues of *bcl-2* have also been detected in the nervous system; *bax* has been detected at the mRNA level in peripheral ganglia of both young and adult rats (Gillardon et al., 1994); and *bcl-x*-deficient mice exhibit extensive neuronal death during neuronal differentiation (Motoyama et al., 1995).

Most recently, a gene encoding neuronal apoptosis inhibitor protein (NAIP) has been isolated and found to be expressed in motor but not sensory neurones (Liston et al., 1996). Like the *bcl* homologues, it appears to be part of a family of genes; detection of a Drosophila homologue would suggest that these genes may be a conserved family of apoptosis supressors (Liston et al., 1996)

The initial studies on the Schwann cell precursors showed rapid death of these cells in the absence of their normal environment and without addition of exogenous growth factors. The manner of death *in vitro* has been investigated and this Chapter describes the evidence for apoptotic death of these cells in the absence of growth factors.

RESULTS

Removal from axonal contact results in rapid death of E14 precursors

It was observed that when E14 Schwann cell precursors were removed from axonal contact and placed in culture in defined medium, almost all did not survive to 20 hr post-plating. As described in Chapter 3, these cells were healthy and flattened at 3 hr post-plating, but by 20 hr only cell debris remained (Figs 3.4, 3.5 A, B). A time course of death was plotted by fixing and immunolabelling cells with p75LNGFr antibodies at 3 hr, 4.5 hr, 6 hr, 7 hr, 9 hr, 11 hr and 20 hr after plating. Survival was calculated as before: the number of flattened p75LNGFr$^+$ cells surviving at a given time was plotted as a percentage of similar cells that had flattened at 3 hr (Fig 4.1).

Cell numbers remained constant until approximately 5 hr after plating. There then followed a period of rapid death, the majority of cells in the cultures were dead by 9 hr after plating and almost all cells were dead after 20 hr in culture. At the end of this time very little cell debris was associated with the coverslip (Fig 3.5 B), unlike the situation found when these cells were killed by toxicity (not shown).

Dying cells exhibit morphological changes and pyknotic nuclei

During the period of cell death in culture, it was observed that the dying cells rounded up and assumed a phase-bright appearance, and often these cells associated with the surface of flattened groups of precursor cells (Fig 4.2 A). The cytoplasm of these cells appeared granular, some cells having vacuoles within the cytoplasm. These shrunken cells stained intensely for p75LNGFr (not shown).

When these cells were stained with the Hoechst nuclear stain H33258 that chelates with the DNA of the cells, the nuclei of the rounded cells appeared to be very small, very intensely stained and were often fragmented into two or more separate (pyknotic) bodies (Fig 4.2 B, arrows). Such nuclear changes indicate nuclear shrinkage, DNA condensation and nuclear fragmentation.

To see whether the cells that were lost from the precursor monolayer during the period of cell death could be detected in the culture medium, cell supernatant from a single experiment at 8 hr post-plating was centrifuged and the pellet fixed before transferring to a nitrocellulose filter. This was then stained with Hoechst dye and viewed using Hoechst optics. Despite the thickness of the filter, the fragmented cell nuclei could be

determined and counted. Approximately 80% of cells that were lost from the monolayer could be recovered in this way.

Dying precursors have fragmented DNA

To further study the fragmentation of the DNA observed with Hoechst dye staining, E14 precursors were cultured in non-conditioned defined medium for 5 and 7 hr, and in NCM for 20 hr. Newborn Schwann cells were cultured in non-conditioned defined medium for 20 hr for a comparison. At the end of the culture time, both floating cells and cells still attached to the culture substrate were lysed, digested and extracted, and the DNA run on a 1.7% agarose gel; with E14 cells cultured in NCM for 20 hr the floating cells and adherent cells were lysed separately. Ethidium bromide was used to detect the DNA under UV light (Fig 4.3). Each treatment of the precursors represents a separate dissection. The number of cells in the starting populations of all the treatments was similar, 400,000- 500,000 cells. The samples were all treated identically to prepare the cell lysates and the volumes loaded onto the gel were identical in all cases.

Fragmentation of the DNA into repeated nucleosome lengths was observed in precursors as early as 5 hr after plating in defined medium (Fig 4.3, lane 1). This laddering was more pronounced by 7 hr (Fig 4.3, lane 2). Precursors cultured in NCM, however, showed no laddering even after 20 hr in culture (Fig 4.3, lane 3), although extremely faint laddering could be detected if the small proportion of floating cells were removed and lysed separately (Fig 4.3, lane 4). Newborn Schwann cells cultured for 20 hr in defined medium showed no laddering of the DNA (Fig 4.3, lane 5).

Protein synthesis inhibitors delay precursor death

The protein synthesis inhibitors cycloheximide and emetine were used to see whether new proteins were required for the death of precursors in defined medium. Cycloheximide at a concentration of 0.5μg/ml or emetine at a concentration of 1.5μg/ml were included in the defined medium added to the cells 3 hr after plating. These concentrations had been determined to be the most effective at prolonging precursor survival (dose response curves not shown here). The survival of cells was determined at 9 hr and 20 hr post-plating by fixing and immunolabelling the cells for

p75LNGFr, and calculating survival as a percentage of the cells at 3 hr as described previously.

Figure 4.4 shows the survival at 3 hr, 9 hr and 20 hr after plating. At 9 hr, only 23% ± 3 (SEM, n=3) of cells were alive without protein synthesis inhibitors. In the presence of 0.5μg/ml cycloheximide, 82% ± 13 (SEM, n=3) were alive, flattened and $p75LNGFr^+$ at this time. Emetine was less effective at preventing precursor death: in defined medium with 1.5μg/ml emetine only 54% ± 7 (SEM, n=3) survived at 9 hr. By 20 hr, only 2% ± 1 (SEM, n=3) of the cells flattened and labelled at 3 hr remained in defined medium alone. The number of cells surviving in cycloheximide decreased to 25% ± 6 (SEM, n=3) and only 7% ± 2 (SEM, n=3) of cells remained in cultures that included emetine. The cells surviving in cycloheximide continued to die over the next 12 hr. These results indicate that, although protein synthesis inhibitors can delay precursor death in defined medium, ultimately these cells will die in the absence of trophic factors.

Single experiments with the protein synthesis inhibitors anisomycin (at 0.5μg/ml) and puromycin (at 1μg/ml) produced 18% and 13% precursor survival respectively, at 9 hr (not shown). By 20 hr, however, less than 1% of cells survived.

Addition of forskolin delays precursor death in a dose-dependent manner

Elevation of cyclic AMP in cultures of post-natal Schwann cells upregulates the expression of the myelin protein P_0 (Morgan et al., 1991) and has been thought to mimic *in vivo* signals from axons to Schwann cells. To see whether elevation of cAMP would promote the surivival of the precursors in culture, two concentrations, 2μM and 20μM, of the cAMP-elevating agent forskolin were used in defined medium and the survival of precursor cells determined at 4.5 hr, 6 hr, 10 hr and 20 hr (Fig 4.5).

Both concentrations of forskolin delayed the death of the precursors with 20μM more effective than 2μM (Fig 4.5). The delay was first obvious at 6 hr post-plating, when 56% ± 7 (SD, n=2) of cells were alive in defined medium but 82% ± 14 (SD, n=4) of cells remained in 2μM forskolin and 92% ± 8 (SD, n=2) of cells were alive in 20μM. By 10 hr, only 11% ± 10 (SD, n=2) of cells survived in defined medium but 36% ± 7 and 68% ±13 (SD, n=2) of cells were alive in 2μM and 20μM forskolin respectively. Only 20μM forskolin had an effect at 20 hr, where 16% ± 16 (SD, n=2) of cells

remained alive, suggesting that elevating cAMP can delay but not prevent precursor death.

Figure 4.1 Time course of precursor death *in vitro*

E14 precursors were fixed and immunolabelled with p75LNGFr after various times in culture, in the presence of nonconditioned defined medium. Survival is expressed as a percentage of flattened p75LNGFr$^+$ cells remaining at each timepoint relative to the number of these cells at 3 hr. There is an initial delay of approximately 4-5 hr before loss of cells is apparent. Death then proceeds rapidly, with most cells dying by 9 hr after plating. Each point represents the average from three experiments, error bars indicate SEM, n=3.

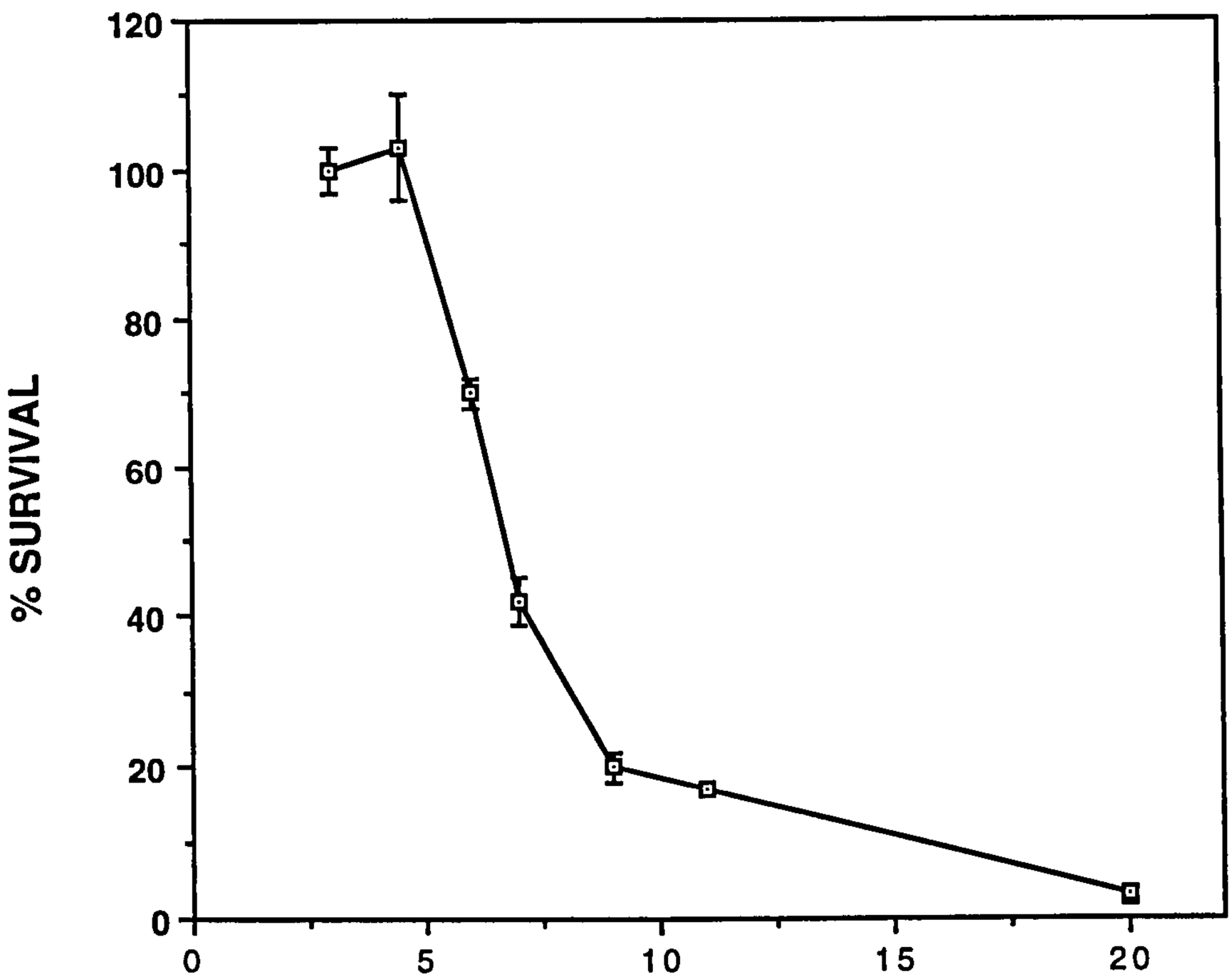

120
100
80
60
40
20
0
% SURVIVAL
0
5
10
15
20
TIME AFTER PLATING (hours)

Figure 4.2 Morphological changes in cultured precursors indicative of apoptosis

(A). Phase-contrast micrograph of precursors cultured for 7 hr in nonconditioned defined medium. Numerous rounded, phase-bright cells are found in association with an area of apparently normal flattened cells. The cytoplasm of these phase-bright cells appears granular, with evidence of vacuoles. Bar = 20μm

(B). The cells shown in (A) stained with Hoechst H33258 dye and viewed with Hoechst fluorscence optics. The rounded cells visible in (A) have small, intensely stained nuclei. In many of these cells the nuclei have fragmented (arrows).

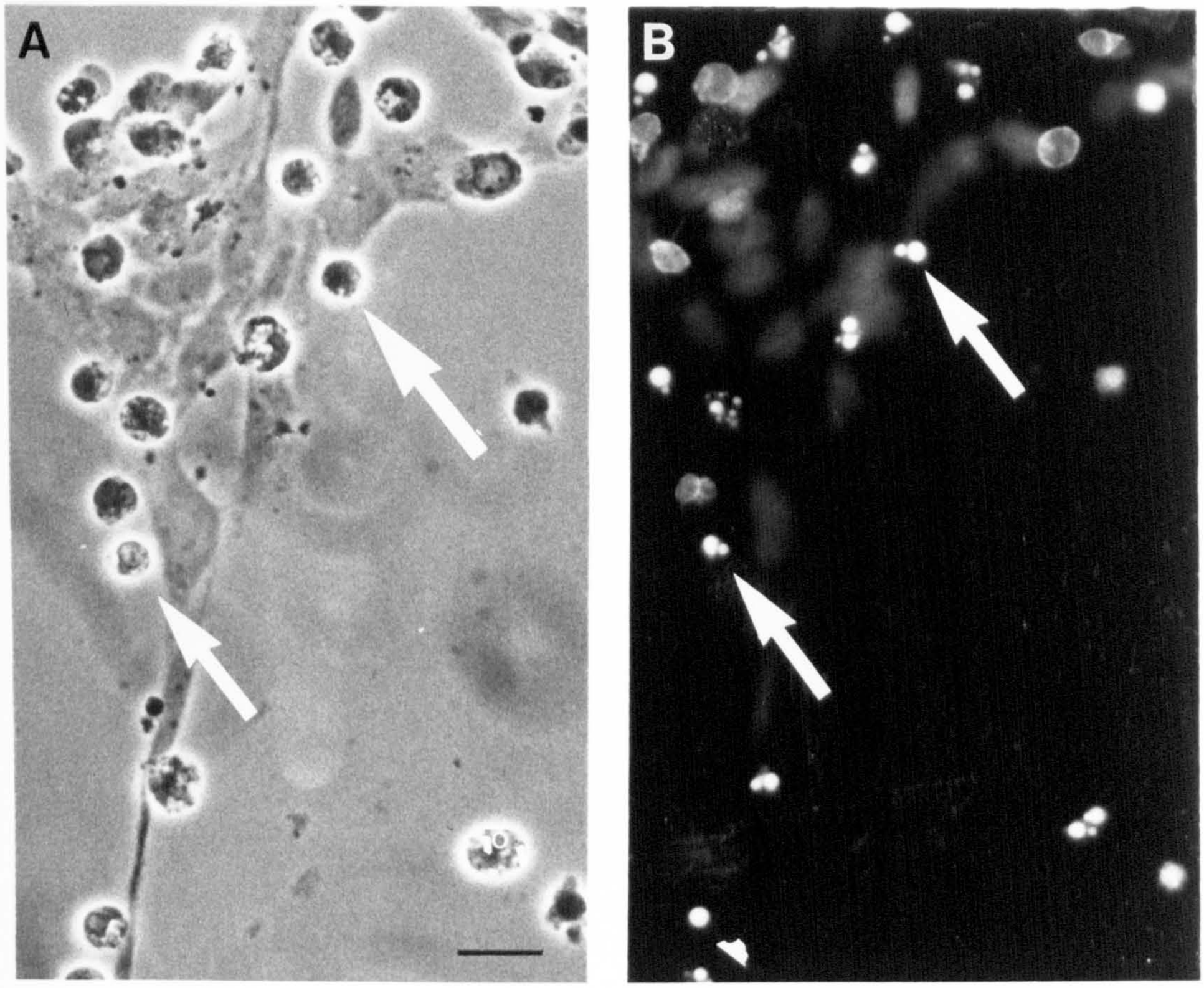
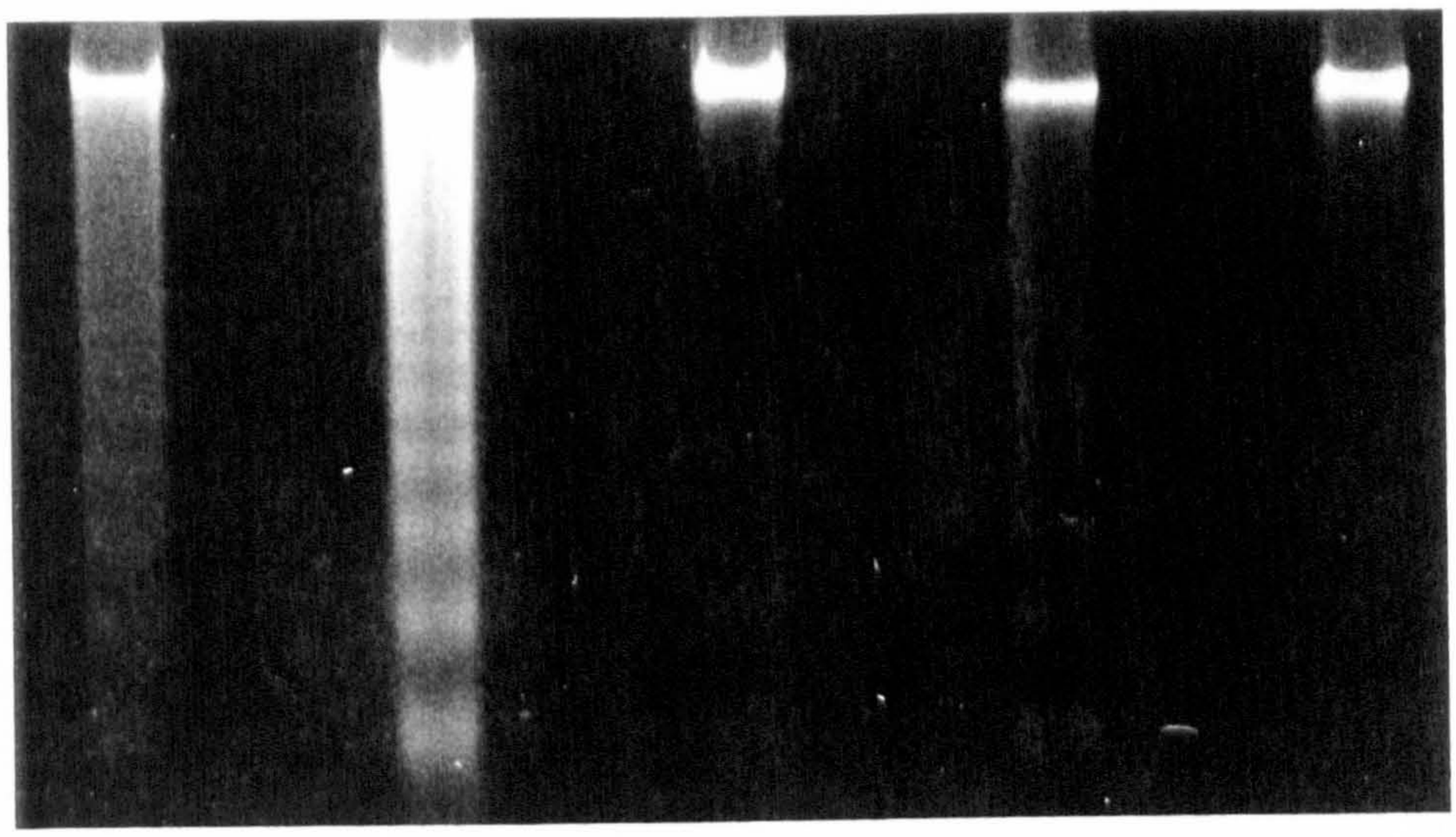

A
B

Figure 4.3 Dying precursors exhibit DNA laddering

A DNA fragmentation assay of E14 precursors (lanes 1-4) and newborn Schwann cells (lane 5). The DNA extracts were run on a 1.7% agarose gel with ethidium bromide and visualized using UV illumination. Laddering of DNA is detectable in precursors cultured in defined medium as early as 5 hr after plating (lane 1). This laddering is intensified after 7 hr culture of these cells in defined medium (lane 2). Culture of E14 precursors in defined medium conditioned by DRG neurones prevents DNA fragmentation even 20 hr after plating (lane 3); floating cells from the same culture exhibit faint DNA laddering (lane 4). Newborn Schwann cells do not exhibit fragmentation when cultured for 20 hr in defined medium alone (lane 5).

1 2 3 4 5



E14
5 hr DM

E14
7hr DM

E14
20 hr NCM

E14
20 hr NCM
FLOATING
CELLS

NB
SCHWANN
CELLS
20 hr DM

Figure 4.4 Inhibitors of protein synthesis delay precursor death in defined medium

Addition of 0.5μg/ml cycloheximide (hatched column) or 1.5μg/ml emetine (grey column) delayed the death of E14 precursors cultured in defined medium. Survival of cells cultured in defined medium alone is represented by the black columns. Cycloheximide is more effective than emetine at delaying precursor death at both 9 hr and 20 hr after plating. Cells were stained with p75LNGFr and counted at the timepoints indicated. Survival is calculated relative to cells at 3 hr as described previously. Each point is the average from three experiments, error bars indicate SEM.

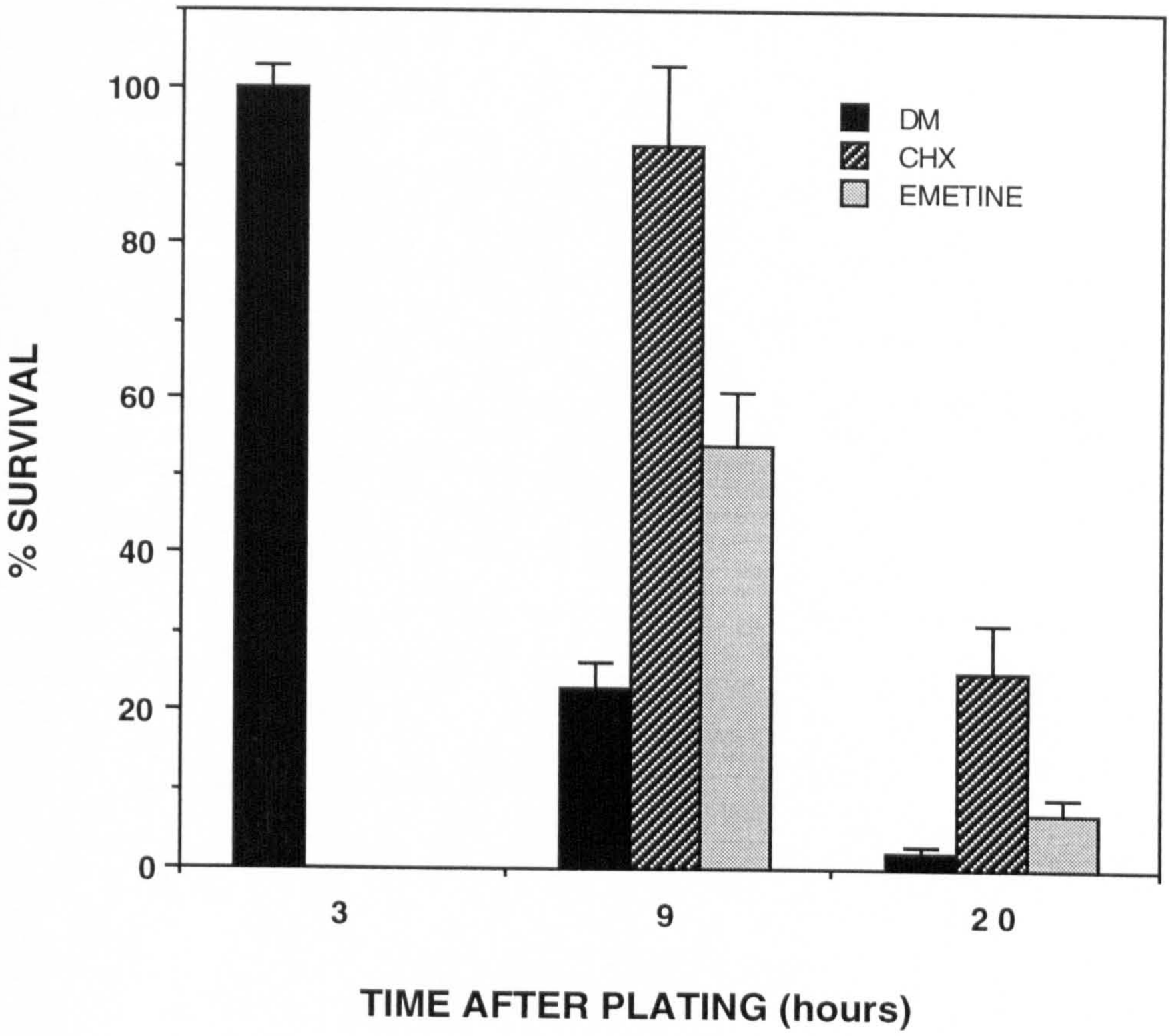
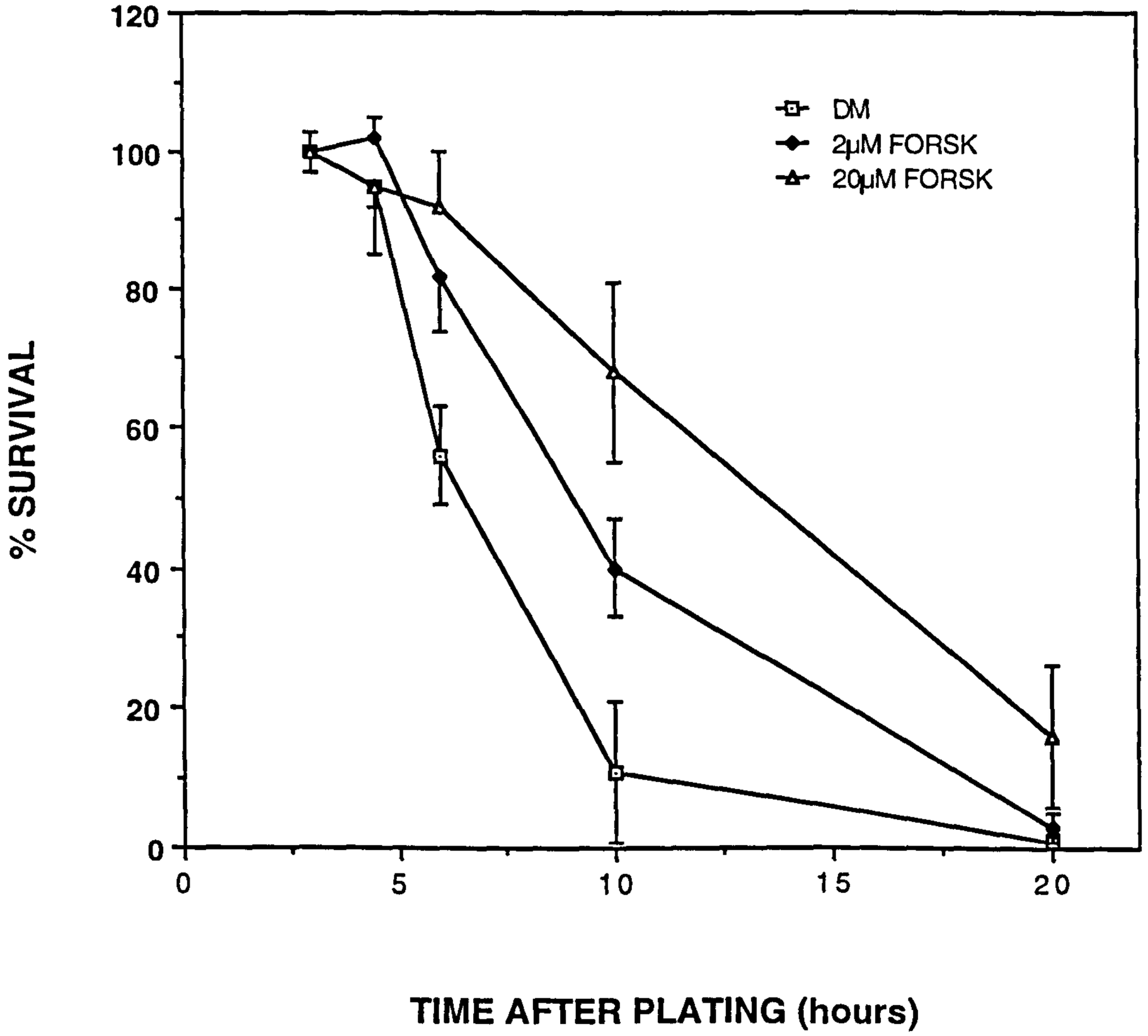

100
80
60
40
20
0
% SURVIVAL
DM
CHX
EMETINE
3
9
20
TIME AFTER PLATING (hours)

Figure 4.5 Forskolin causes a dose-dependent delay in precursor death

Treatment of cells with 2μM forskolin (diamonds) or 20μM forskolin (open triangles) delays the death of E14 precursors. Open squares represent the survival of E14 cells cultured in defined medium alone. 20μM forskolin is more effective and delaying the onset of precursor death. Cells were stained with p75LNGFr at the timepoints indicated and survival calculated as described previously. Each point represents the average from three experiments, error bars indicate SEM.


120
100
80
60
40
20
0
% SURVIVAL
0
5
10
15
20
DM
2μM FORSK
20μM FORSK
TIME AFTER PLATING (hours)

DISCUSSION

Although there is no *in vivo* evidence in the rat for Schwann cell apoptosis during development, it is believed that many, if not all cells will undergo apoptosis when cultured in conditions that deprive them of factors essential to their continued survival (Raff, 1992). The recent observation that chick Schwann cells undergo apoptosis *in vivo* during normal development (Ciutat et al., 1996), suggests that Schwann cell death is a normal event in peripheral nerve development. This chapter provides evidence that Schwann cell precursors exhibit characteristic changes associated with apoptotic death when they are removed from axonal contact and cultured *in vitro* in defined medium.

Morphological changes and the timecourse associated with precursor death *in vitro*

As described earlier, withdrawal of essential growth factors from many cell types results in the initiation of death by apoptosis (Collins et al., 1994). When Schwann cell precursors are removed from axonal contact and placed in culture in defined medium containing high levels (1μM) of insulin, death is rapid. Despite the cells flattening and appearing healthy at 3 hr post-plating (Fig 3.5 A), evidence of cell death is apparent from five hours in culture onwards (Fig 4.1). Rapid cell loss follows until, by 20 hr post-plating, very few p75LNGFr$^+$ cells remain.

On its own, the rapidity of death of these cells is not indicative of apoptosis. Different cell types appear to have varying times of response to apoptotic stimuli: thymocytes subjected to the glucocorticoid dexamethasone show signs of apoptosis by 2 hr after treatment (Cohen and Duke, 1984) while hepatocytes treated with TGFβ1 do not exhibit any significant changes until 20-24 hr after treatment (Oberhammer et al., 1992).

Morphological changes associated with death by apoptosis were observed in the precursors during the period of cell death in culture. The cells withdrew their short cytoplasmic processes and rounded up, appearing as phase-bright images often in association with the precursor monolayers (Fig 4.2 A, arrows). Such cytoplasmic condensation is consistent with apoptosis (Wyllie et al., 1980; Kerr et al., 1987). Granular cytoplasm and the presence of vacuoles within some of these phase-bright

cells are also indicators of apoptosis (Wyllie et al., 1980; Kerr et al., 1987). During these cytoplasmic changes, the nuclei of the precursors could be seen to condense and, in some cases, were observed to be fragmented. Despite the extensive death in these cultures, no cells were observed that exhibited any signs of necrosis: there was no membrane swelling or visible lysis of the cells during the period of cell death.

As described earlier, the earliest morphological event of apoptosis at the nuclear level is chromatin condensation which can only be observed in the electron microscope (Kerr et al., 1987). Chromatin is sharply defined and condensed to the edges of the nucleus (Wyllie et al., 1980). Electron microscopic studies of dying Schwann cell precursors have revealed such nuclear changes, with chromatin condensed into crescents around around the margin of the nucleus (Jessen et al., 1994).

By staining the dying cells with the nuclear dye Hoechst H33258, nuclear condensation and fragmentation was most apparent (Fig 4.2 B). Intensely stained nuclei were observed in all the phase-bright cells, with some of the nuclei having fragmented (Fig 4.2 B arrows). Such changes in nuclear structure are consistent with the process of apoptosis (Wyllie et al., 1980; Kerr et al., 1987).

Apoptosis of cells *in vivo* is followed by rapid phagocytosis of these cells by their neighbours (Wyllie et al., 1980; Kerr et al., 1987). The timecourse of precursor death revealed that cells rapidly disappeared from the coverslips after assuming apoptotic characteristics. Examination of the supernatant revealed that the majority (80%) of these cells could be recovered from the medium, suggesting that phagocytosis was not responsible for their apparent loss, rather that they had lost the ability to adhere to the coverslip or their neighbouring cells. Changes affecting cell adhesion during apoptosis have been reported *in vivo* where cells separate from the surrounding tissue at the time of cytoplasmic condensation, for example: epithelial cells lose desmosomal connections (Wyllie et al., 1980).

DNA fragmentation as a signature of apoptotic death

As the morphological changes described above strongly suggested that the Schwann cell precursors died by apoptosis, an investigation was made of one of the most characteristic events of apoptosis, double stranded DNA fragmentation. Separation of fragmented DNA on agarose gels produces a DNA ladder, first observed in apoptosing

thymocytes by Wyllie (1980). Although not all cells undergoing apoptosis exhibit DNA fragmentation, where fragmentation is present and is associated with other characteristics such as cytoplasmic condensation, it is indicative of apoptosis (reviewed in Bortner et al., 1995).

DNA fragmentation, or laddering, could be detected in Schwann cell precursors as early as 5 hr after plating in defined medium (Fig 4.3, lane 1), with an increase in intensity of the bands by 7 hr post-plating (Fig 4.3, lane 2). Conditioning of the medium by neurones abrogated this effect, with no laddering being detected in adherent cells at 20 hr post-plating (Fig 4.3, lane 3). Laddering, however, was detected in the floating cells found in the NCM-treated cultures (Fig 4.3, lane 4). Schwann cells from newborn nerves cultured in defined conditions showed no laddering at 20 hr post-plating (Fig 4.3, lane 4).

The timing of the appearance of DNA fragmentation in the precursors cultured in defined medium correlates well with the first morphological changes associated with the death of these cells, just preceding the loss of cells from the coverslips (first apparent five hours post-plating). Cell loss in the cultures reaches about 50% of the starting population at 7 hr after plating and this is reflected by an increase in intensity of the DNA bands.

The absence of DNA laddering in adherent precursors cultured in NCM is consistent with the survival of these cells in culture to 20 hr post-plating and beyond. This shows that factors in the NCM can suppress apoptosis in these cells. As reported in Chapter 3, NCM promotes the survival of 80% of precursors at 20 hr post-plating. When floating cells from the precursor cultures were examined for DNA fragmentation faint bands could be detected, suggesting that the 20% of cells that are not rescued by NCM die by apoptosis. These cells may be unable to respond to the survival factors present in the NCM either because they lack sufficient receptors for the factors (Collins et al., 1994), or because they have already irreversibly committed to the apoptotic pathway prior to addition of NCM to the cultures.

The absence of DNA laddering in newborn Schwann cells cultured under the same conditions that produced laddering in E14 precursors, would indicate that these older cells are capable of producing their own survival factors, either acting in a paracrine or an autocrine fashion (discussed in Chapter 3).

The DNA ladder seen on the agarose gel indicates internucleosomal cleavage of the DNA. It is thought that an endogenous endonuclease cleaves the DNA in the linker region between the histones on the chromosomes (Wyllie, 1980; reviewed in Bortner et al., 1995). The DNA wrapped around each histone is approximately 180-200 base pairs, thus the ladder represents multiples of this unit (Wyllie, 1980; Bortner et al., 1995, Earnshaw, 1995a). This fragmentation of the DNA is an early event in the process of apoptosis and is irreversible, once DNA has been cleaved in this manner the cell will die (Bortner et al., 1995).

The enzyme responsible for the DNA fragmentation has yet to be clearly identified, and it is possible that a different nuclease is responsible for this phenomenon in different cell types (Earnshaw, 1995a). Early studies on thymocyte apoptosis point to the action of a calcium-dependent endonuclease in DNA fragmentation (Wyllie, 1980; Cohen and Duke, 1984). But other enzymes have been identified as fragmentation nucleases including DNase I, DNase II and cyclophilin A (reviewed by Bortner et al., 1995; Earnshaw, 1995a).

Although no studies have been performed on the nature of the nuclease involved in Schwann cell precursor apoptosis, the timing of the appearance of the calcium-binding protein S100 is closely correlated with the ability of the Schwann cell precursors to survive culture in defined medium (as discussed in Chapter 3) and it is interesting to speculate that modulation of calcium in these cells could reduce endogenous activity of a calcium-dependent endonuclease. As described in Chapter 6, however, maturation of the Schwann cell precursors in growth factors produces some cells that appear to live in defined medium without detectable S100 which would suggest either that modulation of calcium levels within these cells is not the sole factor controlling apoptosis.

Protein synthesis inhibitors can delay precursor death

As described earlier, death by apoptosis is an active process. Both RNA and protein synthesis inhibitors have been shown to suppress apoptosis in many cells including thymocytes (Cohen and Duke, 1984; Wyllie et al., 1984), oligodendrocytes (Barres et al., 1992) and neurones both *in vivo* (Oppenheim et al., 1990) and *in vitro* (Martin et al., 1988; Scott and Davies, 1990; Edwards et al., 1991).

Delay of apoptosis in the Schwann cell precursors by the protein synthesis inhibitors cycloheximide and emetine suggests that the process of death in these cells requires *de novo* protein synthesis. However, the protein synthesis inhibitors did not promote long term survival.

Although RNA and protein synthesis inhibitors have been shown to delay or block the death of many cell types as mentioned above, the reverse of this situation is true in other cells. For example, protein synthesis inhibitors initiate apoptosis in metamyelocytes (Cohen, 1991), the promyelocytic leukaemia cell line HL-60 (Martin et al., 1990) and macrophages (Waring, 1990). The induction of apoptosis in such cells has been put forward as evidence for the ubiquitous presence of proteins that initate the process of apoptosis (see below) (Raff, 1992; Steller, 1995). Support for this idea comes from a system where enucleated cells can still undergo apoptosis following growth factor withdrawal (Jacobsen et al., 1994).

As described above, cells appear to contain both repressors (bcl-2, bcl-x, bax, NAIP) and activators (ICE, Ich-1, CPP32, *reaper*) of apoptosis (Hockenbery et al., 1990; Kumar et al., 1994; Merry et al., 1994; Wang et al., 1994; White et al., 1994; Motoyama et al., 1995; Nicholson et al., 1995; Liston et al., 1996; Middleton et al., 1996). The relative levels of these apoptotic modulators and the rate of their turnover may influence what happens to a cell when it is exposed to adverse conditons such as growth factor withdrawal. It has been suggested that RNA and protein synthesis may be required to produce derepressors of the cell death pathway rather than molecules directly involved in the death process (Steller, 1995). In cells where inhibition of macromolecular synthesis induces apoptosis, however, it may be that the repressors are found at low levels and are continuously turned over; blocking synthesis of new repressors may alter the balance of the apoptotic proteins towards activation of the death pathway.

Protein synthesis inhibitors delay Schwann cell precursor death but do not prevent these cells from ultimately dying by apoptosis. A similar situation is found in oligodendrocytes (Barres et al., 1992) but neurones exhibit long term survival after inhibition of macromolecular synthesis (Martin et al., 1988; Scott and Davies, 1990; Oppenheim et al., 1990; Edwards et al., 1991). This would suggest that the control of apoptosis within neurones and glia is different.

The different effects of protein synthesis inhibitors on preventing apoptosis have been reported in other cells, for example rat thymocytes (Chow et al., 1995). Treatment of these cells with methylprednisolone, ionomycin or thapsigargin results in DNA fragmentation which is inhibited by cycloheximide and partially inhibited by emetine. Puromycin, however, actively induces DNA fragmentation even at concentrations that greatly inhibited protein synthesis (Chow et al., 1995).

The protein synthesis inhibitors used in the current study all have different modes of action (Pestka, 1971) but it is possible that they have other activities that are also able to interfere with apoptosis. Cycloheximide and anisomycin are reported to superinduce immediate-early genes (Edwards and Mahadevan, 1992; Zinck et al., 1995) and to activate intracellular signalling pathways involving mitogen-activated protein kinase (MAPK) and stress-activated protein kinase (SAPK) (Zinck et al., 1995). However, emetine and puromycin do not produce the same effects (Edwards and Mahadevan, 1992). It is unlikely that the action of protein synthesis inhibitors in delaying or preventing cell death occurs via superinduction of immediate-early genes: the immediate-early genes *c-fos* and *c-jun* have been implicated in postive regulation of apoptosis (Smeyne et al., 1993; Ham et al., 1995). However, transgenic mice have been described that lack *c-jun*, *c-fos*, and both *c-jun* and *c-fos* (Roffler-Tarlov et al., 1996); the mice do not exhibit unusual patterns of cell death suggesting that these immediate early genes are not essential for apoptosis.

The cAMP-elevating agent forskolin delays precursor death in a dose-dependent manner.

Elevation of intracellular cAMP *in vitro* can to some extent mimic the action of axon-associated signals on Schwann cells inducing either proliferation or myelination depending on the conditions (Sobue et al., 1986; Lemke and Chao, 1988, Monuki et al., 1989; Mirsky et al., 1990; Weinmaster and Lemke, 1990; Morgan et al., 1991). In other culture systems, cAMP analogues have been reported to act as survival factors for rat sympathetic and sensory neurones in the absence of NGF (Rydel and Greene, 1988; Edwards et al., 1991). In these systems, the cAMP analogues mimicked NGF, promoting long term survival of the neurones. Sympathetic neurones could be rescued even after initiation of the death program with both cAMP analogues and NGF (Edwards et al., 1991). This rescue did not require *de novo* protein synthesis and it

was suggested that both cAMP elevation and NGF may act on a common target or related targets, possibly by post-translationally modifying proteins involved in the death pathway (Edwards et al., 1991).

cAMP elevation, like protein synthesis inhibition, did not promote long term survival in the Schwann cell precursors, again revealing differences between neurones and glia in the control of apoptosis.

Unlike differentiated neurones, the Schwann cell precursor is a highly motile cell (Jessen et al., 1994). The presence of a sensitive mechanism for killing these cells should they stray from their normal environment might therefore be important. If it is assumed that the axons are the source of precursor survival factors *in vivo*, the ability of these cells to undergo apoptosis rapidly after growth factor withdrawal would constitute an essential control in normal peripheral nerve development.

In conclusion, Schwann cell precursors undergo death when removed from axonal contact. This process is rapid, with half the cells dying 7 hr after plating in defined medium and essentially all cells dying by 20 hr in culture. Morphological and nuclear changes indicate that death is by apoptosis. Death of these cells can be delayed but not prevented by protein synthesis inhibitors or by the cAMP-elevating agent forskolin.

CHAPTER 5

FGFS AND IGFS ARE REQUIRED FOR SHORT TERM SCHWANN CELL PRECURSOR SURVIVAL

INTRODUCTION

The requirement of cells in culture for exogenous growth factors has been well documented in many systems, including oligodendrocytes (Barres et al., 1993) and neurones of the peripheral nervous system (Thoenen, 1991; Snider, 1994). The Schwann cell precursors are able to survive in culture if the medium has been conditioned by DRG neurones, implying that neurones are a source of trophic factors for these cells. Earlier studies showed that the factor or factors were sensitive to trypsin and heparin (Jessen et al., 1994) and that bFGF, a heparin-binding growth factor could support the survival of precursors in the presence of high concentrations of insulin (Jessen et al., 1994). Studies described in this chapter examine the ability of a large number of growth factors, including other members of the FGF family to rescue precursors *in vitro* (Gavrilovic et al., 1995).

Nine members of the fibroblast growth factor family have been identified so far; acidic FGF (FGF-1) and basic FGF (FGF-2) being the first members of the family to be isolated (reviewed in Basilico and Moscatelli, 1992; Baird, 1994). All the members share a degree of homology, with aFGF and bFGF being 55% homologous at the protein level (Basilico and Moscatelli, 1992). These peptides have similar molecular weights of 15.5-17 kD, but higher molecular weight forms of bFGF have been reported in some tissues including the brain, (Basilico and Moscatelli, 1992). K-FGF (FGF-4) is 43% homologous to bFGF and is somewhat larger, with a molecular weight of 22-23 kD (Delli-Bovi et al., 1988). Non-glycosylation of K-FGF during its synthesis results in its immediate cleavage into two NH_2-terminally truncated fragments of 13 and 15 kD, the latter fragment, referred to as K140, possessing five-times the activity of the parent K-FGF molecule in mitogenic assays using 3T3 fibroblasts (Bellosta et al., 1993).

Both aFGF and bFGF, together with the latest addition to the FGF family, FGF-9, lack a conventional signal sequence, making their release from cells a matter for debate (Basilico and Moscatelli, 1992; Mason, 1994). It is thought that cells can release small amounts of the factors by an unknown novel secretory mechanism. The fact that aFGF and bFGF are found in serum and medium from cultured cells would support this idea (Mason, 1994).

FGFs are potent mitogens for many tissues and have angiogenic properties (Gospodarowicz et al., 1987; Basilico and Moscatelli, 1992). They have also been implicated in tissue patterning of the embryo especially during gastrulation and limb formation, and in tissue differentiation (reviewed in Yamaguchi and Rossant, 1995). During development, FGFs and their receptors are expressed in a cell-specific manner, some of the members such as bFGF and aFGF having a broad distribution, while other members are highly restricted (Basilico and Moscatelli, 1992; Givol and Yayon, 1992; Yamaguchi and Rossant, 1995).

Four separate genes have been identified for the high affinity FGF receptors, designated FGFR1-4. These receptors are protein tyrosine kinases that share a common structure, with three extracellular Ig loops in the ligand-binding domain (reviewed in Givol and Yayon, 1992). On binding ligand, the receptors dimerize and the intracellular kinase domains are activated, producing autophosphorylation and phosphorylation of cytoplasmic substrates such as phospholipase Cγ (Basilico and Moscatelli, 1992). Truncated isoforms of the receptors, lacking either the transmembrane or the intracellular kinase domain are also found, although it is not clear what function they perform (Givol and Yayon, 1992). The heparan sulfate proteoglycans (HSPGs) have been referred to as the low affinity FGF receptors (reviewed in Baird, 1994). The binding of the FGFs to their high affinity receptors is greatly enhanced by FGF binding to HSPGs on the cell surface or in the surrounding extracellular matrix (Mason, 1994); similarly, addition of exogenous heparin to cells in culture can facilitate the binding of FGFs to their receptors (Mason, 1994). Spivak-Kroizman et al. (1994) have reported that FGF molecules oligomerize in the presence of heparin, thus leading to FGF receptor dimerization and subsequent activation.

The FGF family members bind with different affinities to the different FGFRs. Alternate splicing of the third Ig domain of all the receptors produces variants with altered ligand affinities and specificities, and these alternate forms are expressed in a tissue-specific manner (reviewed in Givol and Yayon, 1992; Eckenstein, 1994). The binding affinities of members of the FGF family for the full-length FGF receptors have been described as follows: aFGF and bFGF bind with equal high affinity to FGFR1 (0.1-0.5 nM) but K-FGF exhibits 15-fold lower affinity for this receptor (Mansukhani et al., 1990); aFGF, bFGF and K-FGF bind to FGFR2 with equally high

affinities (Mansukhani et al., 1990); FGFR3 binds aFGF preferentially, bFGF exhibits approximately 20-fold lower affinity for this receptor; FGFR4 binds aFGF with similar affinity as that of FGFR1 (Givol and Yayon, 1992).

There is evidence that both aFGF and bFGF can translocate to the nucleus and affect gene transcription directly by an unknown mechanism (reviewed in Prochiantz and Théodore, 1995; Walicke and Baird, 1991). Alternately spliced variants of aFGF and bFGF contain putative nuclear localization sequences (Jans, 1994). This offers a potential intracrine pathway in cells that produce FGFs, and an alternative mechanism of action to the conventional signal transduction sequence when FGFs act in a paracrine manner.

As described earlier, bFGF has been reported to affect cells of the neural crest, promoting the survival of nonneuronal cells derived from chick trunk neural crest (Kalcheim, 1989) and rat trunk neural crest cells (Bannerman and Pleasure, 1993); this factor enhances the development of melanocytes from chick neural crest cells in culture (Stocker et al., 1991). For Schwann cells, bFGF is a mitogen in the presence of forskolin (Stewart et al., 1991; Schumacher, 1993).

In order to determine other potential survival factors for the Schwann cell precursors, an extensive list of growth factors were tested in the survival assay. The observations of the survival effects of aFGF, bFGF, and K-FGF were extended to determine the required combination of factors to promote the full survival of the precursors. The results of these studies are described in this Chapter.

RESULTS

The effect of growth factors on precursor survival

An extensive list of growth factors was screened for their ability to promote the survival of the Schwann cell precursors in the standard 20 hr survival assay (Table 5.1). The factors were used in defined medium containing 5.7μg/ml (1μM) insulin or 100ng/ml (13nM) IGFs, some factors were combined with the cAMP elevating agent forskolin. The concentration ranges used were those shown to have biological activity in other systems.

Most growth factors did not support survival under any of the conditions used, survival was less than 10% in these cases. Factors that supported significant survival were aFGF, bFGF and K-FGF, three members of the endothelin family, ET-1, ET-2 and ET-3, and NDFβ_2, a member of the neuregulin family of growth factors. All the growth factors except NDFβ_2 required the presence of insulin and forskolin or IGFs for their effects. The results of studies with FGFs are described below, the neuregulin and endothelin results are described in Chapter 6.

bFGF-mediated rescue of E14 precursors depends on IGF receptor activation

It was observed in defined medium that 3ng/ml (0.182nM, MW 17 kD) bFGF, in the presence of 5.7μg/ml (1μM) insulin, promoted the survival of 50% of precursors at 20 hr. Omission of insulin from this medium resulted in death of most of the cells (Fig 5.1).

In order to study the effects of insulin and IGFs on the action of bFGF, cells were plated out in a constant concentration (3ng/ml, 0.182nM) of bFGF without insulin or IGFs. At 3 hr, defined medium containing 3ng/ml (0.182nM) of bFGF and increasing concentrations of either insulin, IGF-1 or IGF-2 were added to the cells; dose response curves of survival at 20 hr were plotted (Fig 5.1). The survival effects of bFGF and IGF on precursors at 20 hr post-plating can be seen in Fig 5.2.

All three insulin growth factors promoted precursor survival in a dose-dependent manner. However, insulin in the presence of bFGF promoted survival of only 50% of the cells, even at a concentration of 5.7μg/ml (1μM). This concentration is believed to be high enough to interact effectively with the type 1 IGF receptor (reviewed by Rechler and Nissley, 1985; Neely et al., 1991). Raising the concentration of insulin to

25μg/ml (4μM) in the presence of bFGF resulted in 10% fewer precursors surviving at 20 hr (n=3, data not shown).

IGF-2, in the presence of bFGF, maximally generated 80% survival at a concentration of 50ng/ml, whereas IGF-1 generated 100% survival at the same concentration. Half-maximally effective concentrations (EC_{50}) were thus 20ng/ml, ~3.5nM for insulin, 8ng/ml, ~1nM for IGF-2 and 1ng/ml, ~0.13nM for IGF-1.

In subsequent experiments, IGF-1 was used in preference to IGF-2 since IGF-1 showed a stronger survival effect when combined with bFGF.

IGF-1 requires bFGF to prevent Schwann cell precursor death

To determine whether IGF-1 could promote Schwann cell precursor survival on its own, cells were plated and cultured in 100ng/ml (13nM) IGF-1 alone. Survival of less than 5% of the cells flattened at 3 hr was seen at 20 hr under these conditions. However, when IGF-1 was combined with increasing concentrations of bFGF survival increased in a dose-dependent manner (Fig 5.3). 100% survival was obtained at 1ng/ml (0.06nM) bFGF and the EC_{50} was ~0.07ng/ml, (0.004nM).

Acidic FGF and K-FGF also support precursor survival in the presence of an insulin growth factor

Two other members of the FGF family, aFGF and K-FGF were assayed for their ability to promote precursor survival in the standard assay. As with bFGF, both these factors were able to act as survival factors for the precursors in the presence of insulin growth factors. Dose-response curves for both these growth factors were generated in the presence of a constant IGF-1 concentration (100ng/ml, 13nM) (Fig 5.4 A) or constant insulin concentration (5.7μg/ml, 1μM) (Fig 5.4 B). The addition of heparin (20μg/ml) to the culture medium was necessary for the action of both aFGF and K-FGF but was not a requirement for the action of bFGF.

Both aFGF and K-FGF were capable of promoting survival of 100% of cells when they were combined with IGF-1 (Fig 5.4 A). The EC_{50} for aFGF was 6ng/ml (~0.39nM, MW 15.5 kD) and for K-FGF 3 ng/ml (~0.14 nM, MW 22 kD).

When a constant concentration of insulin was used, aFGF maximally generated 50% survival at a concentration of 30ng/ml (~1.95nM) (Fig 5.4B), similar to that seen with

bFGF in the presence of insulin (Fig 5.1). However, K-FGF in the presence of insulin maximally generated only 20% survival even at the highest concentration of 100ng/ml (~4.55 nM) (Fig 5.4 B).

Elevation of cAMP promotes precursor survival in bFGF and insulin but does not affect survival in bFGF and IGF-1 or IGF-2

Elevation of cAMP is known to synergize with several growth factors to promote DNA synthesis in Schwann cells. To determine whether cAMP would synergize with the growth factors that promoted precursor survival, the cAMP elevating agent forskolin was used in the survival assays at a concentration of 5μM.

The addition of forskolin to precursors cultured in defined medium containing either 5.7μg/ml (1μM) insulin, 100ng/ml IGF-1 or IGF-2 alone did not promote survival of the cells. The dose-response curves of bFGF with IGF-1 or IGF-2 in the presence of forskolin showed similar maxima for cell survival (Fig 5.5 A, B, C). The dose-response curves were only slightly altered to the left when increasing doses of IGF-1 or IGF-2 were used in constant bFGF (Fig 5.5 A, B) as was that of bFGF in constant IGF-1 (Fig 5.5 C).

In contrast, the effect of insulin in the presence of constant bFGF, and the effect of bFGF in the presence of constant insulin both showed marked enhancement of survival when forskolin was present (Fig 5.6 A, B). In constant bFGF (3ng/ml), maximal survival was increased in 5.7μg/ml (1μM) insulin from ~50% to 100% in the presence of 5μM forskolin (Fig 5.6 A). However, the EC_{50} value for insulin remained unchanged at ~3.5nM (20ng/ml). In constant insulin (5.7μg/ml, 1μM), the maximal survival effect with bFGF was similarly increased in the presence of 5μM forskolin, but the concentration of bFGF required to promote maximal survival was reduced from 3ng/ml to 0.3ng/ml (Fig 5.6 B). The EC_{50} for bFGF was reduced by almost five-fold under these conditions, from ~0.02nM to ~0.004nM, similar to that seen with bFGF in the presence of IGF-1. When the cAMP analogues dibutyryl cAMP and 8-bromo cAMP were used, similar increases in maximal survival were seen, confirming that these effects were due to the elevation of cAMP (data not shown).

Forskolin can synergize with aFGF, K-FGF and K140, in the presence of insulin, to promote survival

Similar experiments were undertaken with aFGF, K-FGF and the truncated, non-glycosylated variant of K-FGF: K140. The effect of 5μM forskolin on the dose-response curves was determined in constant insulin (5.7μg/ml, 1μM) (Fig 5.7 A, B, C). In all cases, the maximum survival of the precursors at 20 hr was increased and the FGF EC_{50} was decreased. In combination with insulin and forskolin, all three factors could support 100% survival at 20 hr; with insulin alone this was maximally 40% with aFGF and only 20% with K-FGF and K140. The EC_{50} values decreased from ~0.36nM to ~0.03nM for aFGF, from ~0.91nM to ~0.182nM for K-FGF and from ~0.30nM to ~0.10nM for K140 in the presence of forskolin.

The action of forskolin with FGFs and insulin does not induce DNA synthesis

Forskolin in combination with FGFs and insulin does not induce DNA synthesis in Schwann cell precursors. Using BrdU incorporation for 8 hr at 12 hr post-plating and at 20 hr post-plating, the level of DNA synthesis in precursors and in newborn Schwann cells was determined. In the presence of bFGF (3ng/ml), insulin (5.7ng/ml) and forskolin 5μM, BrdU incorporation in precursors was 1.0% ± 0.1 (SD, n=2) at 12 hr + 8 hr and 0.4% ± 0.4 (SD, n=2) at 20 hr + 8 hr. Experiments with aFGF and K-FGF under the same conditions gave similar results at 20 hr + 8 hr. For newborn Schwann cells, BrdU incorporation was 22% ± 6 (SD, n=2) at 20 hr + 8 hr.

Do any of the hormones present in the defined medium affect precursor survival?

The defined medium used in all survival assays contained hormones shown to influence cell survival in other systems (Barres et al., 1993): namely dexamethasone, progesterone, thyroxine (T4) and triiodothyronine (T3). To determine whether any of these hormones were promoting survival of the precursors, each factor was omitted from the medium in turn, and also all four were omitted together. The standard assay with bFGF (3ng/ml) and IGF-1 (100ng/ml) was used and survival determined at 20 hr in two separate experiments (Fig 5.8). Using the Student's *t* test, there was no significant difference between survival in most of the different media, the exception being in the omission of triiodothyronine in the presence of bFGF and IGF-1. In this case, the lower survival was statistically significant, with $P < 0.01$. However, when all

four hormones were omitted, the difference in survival was not statistically significant ($P < 0.5$). The effect of triiodothyronine omission on survival was not seen in one experiment when bFGF and insulin were present together (not shown). In the presence of forskolin, no significant difference in survival was seen in any of the media.

Fibronectin can substitute for laminin as a plating substrate for precursors

The effect of the substrate on precursor attachment and survival was studied by plating 4000 cells in defined medium with 5.7μg/ml insulin on coverslips coated with PLL alone, rat tail collagen (10μg/ml) on PLL, fibronectin (25μg/ml) on PLL, and laminin (20μg/ml) on PLL. The cells were then cultured with bFGF (1ng/ml), in the presence of insulin (5.7μg/ml), with or without 5μM forskolin for 20 hr.

On PLL alone, fewer cells attached and flattened at 3 hr compared to laminin: 1764 ± 216 (SEM, n=3) in PLL compared to 2449 ± 248 (SEM, n=3). When cultured in bFGF (1ng/ml), both in the presence and absence of forskolin, survival was never greater than 10% at 20 hr on PLL alone. Cells plated on collagen died before they could attach and flatten, this might be due to impurities in the collagen.

In contrast, precursors attached to fibronectin in similar numbers to those seen on laminin at 3 hr. These cells, in the presence of 5.7μg/ml insulin, showed a similar dose-response to bFGF at lower concentrations of bFGF, both with and without forskolin, as precursors plated on laminin. At 1ng/ml bFGF, a dose producing maximal survival on laminin, there was lower survival on fibronectin at 20 hr. In the absence of forskolin, this difference was not significant ($P < 0.1$; Student's *t* test), but in the presence of forskolin the difference was just significant ($P < 0.05$; Student's *t* test). The average of two dose-response curves ± forskolin on fibronectin is shown in Fig 5.9, together with a dose-response curve for bFGF ± forskolin on laminin from five experiments (previously shown in Fig 5.6B)

FGFs in the presence of IGFs or insulin do not promote the long term survival of precursors

When Schwann cell precursors were cultured in bFGF (3ng/ml) in the presence of 100ng/ml IGF-1, 100% survival was seen at 20 hr. If the culture period was extended to 44 hr, this level of survival dropped to 28% of the population attached at 3 hr, even

if the culture medium was replaced with fresh defined medium containing newly thawed growth factors (Fig 5.10).

Table 5.1 Screen of candidate survival factors for E14 rat Schwann cell precursors

The agents were tested in a 20 hr survival assay on LN-coated glass coverslips. The cells were stained for p75LNGFr. In all cases where survival is indicated as negative, no more than 10% of p75LNGFr$^+$ cells found at 3 hr post-plating were alive at 20 hr.

Comment: (A) Tested in the presence of 1μM insulin in defined medium. (B) Tested as in (A), with and without 5μM forskolin. (C) Tested as in (A) but also in medium containing 50ng/ml IGF-1; both conditions were tested with and without 5μM forskolin.

Table 5.1 Screen of candidate survival factors for E14 rat Schwann cell precursors

Agent	Concentration	Survival	Comment
IGF-1	0.01-100 ng/ml	–	C
IGF-2	0.01-100 ng/ml	–	C
Insulin	0.005-10,000 ng/ml	–	C
PDGF-AA	0.3-30 ng/ml	–	C
PDGF-BB	0.5-50 ng/ml	–	C
NGF	20-2000 ng/ml	–	B
BDNF	0.2-20 ng/ml	–	A
NT-3	0.1-10 ng/ml	–	C
CNTF	2-10 ng/ml	–	C
LIF	10-100 ng/ml	–	B
SCF (Steel factor)	1-100 ng/ml	–	B
α-MSH	10-100 nM	–	A
TPA	5-50 nM	–	A
Ca^{2+}-ionophore, A23187	0.01-100 nM	–	A
TGF-β1	0.1-10 ng/ml	–	A
TGF-β2	0.1-10 ng/ml	–	A
Basic FGF	0.01-10 ng/ml	+	C
Acidic FGF	0.01-100 ng/ml	+	C

Table 5.1 Screen of candidate survival factors for E14 rat Schwann cell precursors

Agent	Concentration	Survival	Comment
K-FGF	0.01-100 ng/ml	+	C
K140 (K-FGF variant)	0.01-100 ng/ml	+	C
ET-1	0.001-100 nM	+	C
ET-2	0.001-100 nM	+	C
ET-3	0.001-100 nM	+	C
NDF-β2	0.1-30 ng/ml	+	C

Figure 5.1 Insulin or IGFs are required for bFGF-mediated rescue of Schwann cell precursors

Results of a 20 hr survival assay in the presence of a constant concentration (3ng/ml) of bFGF. Cell were stained for p75LNGFr at 20 hr and survival calculated as described in Chapter 2. All three insulin growth factors promote precursor survival in bFGF in a dose-dependent manner. The points represent an average of three experiments for IGF-1 and IGF-2, and five experiments for insulin, error bars indicate SEM.

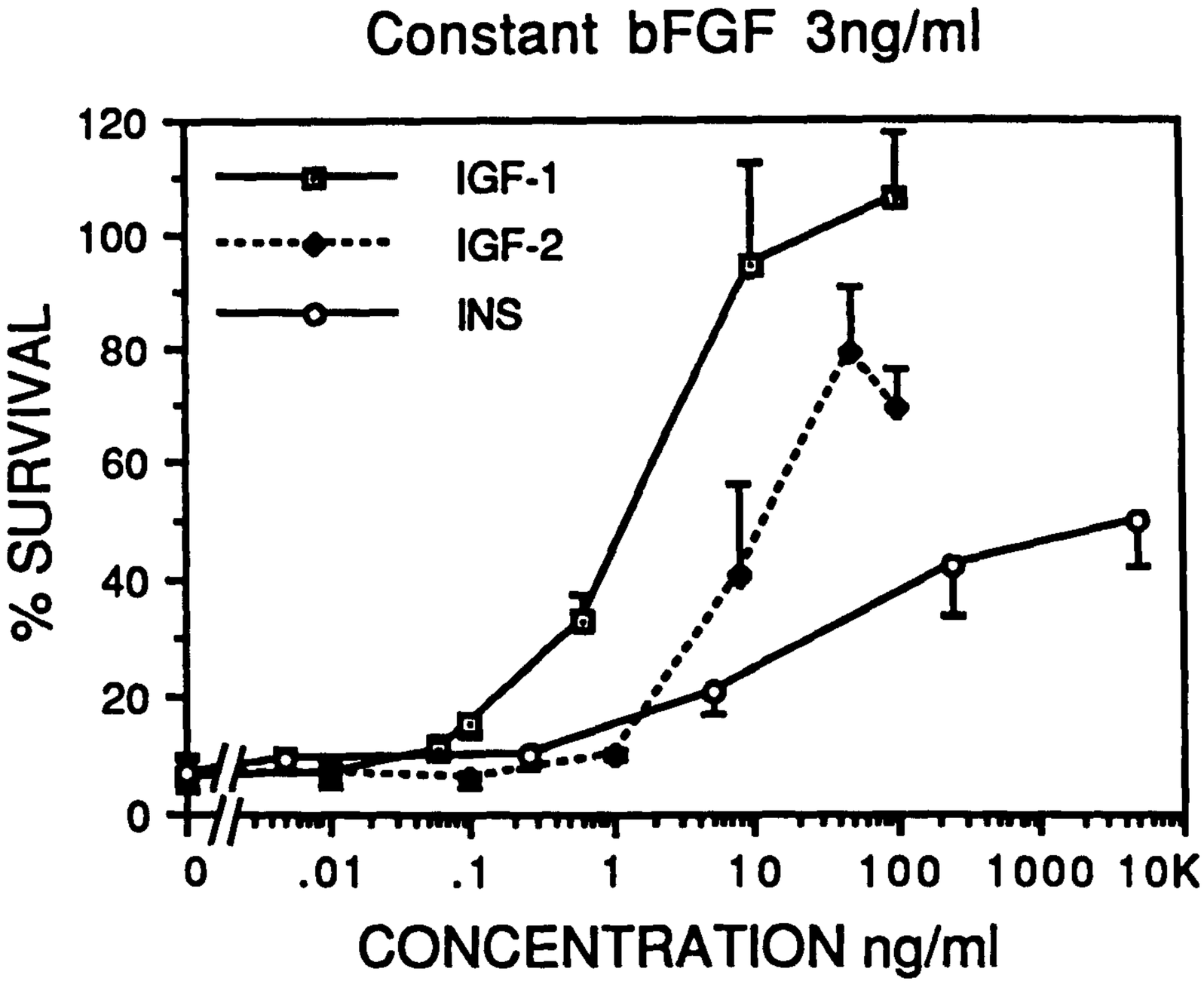
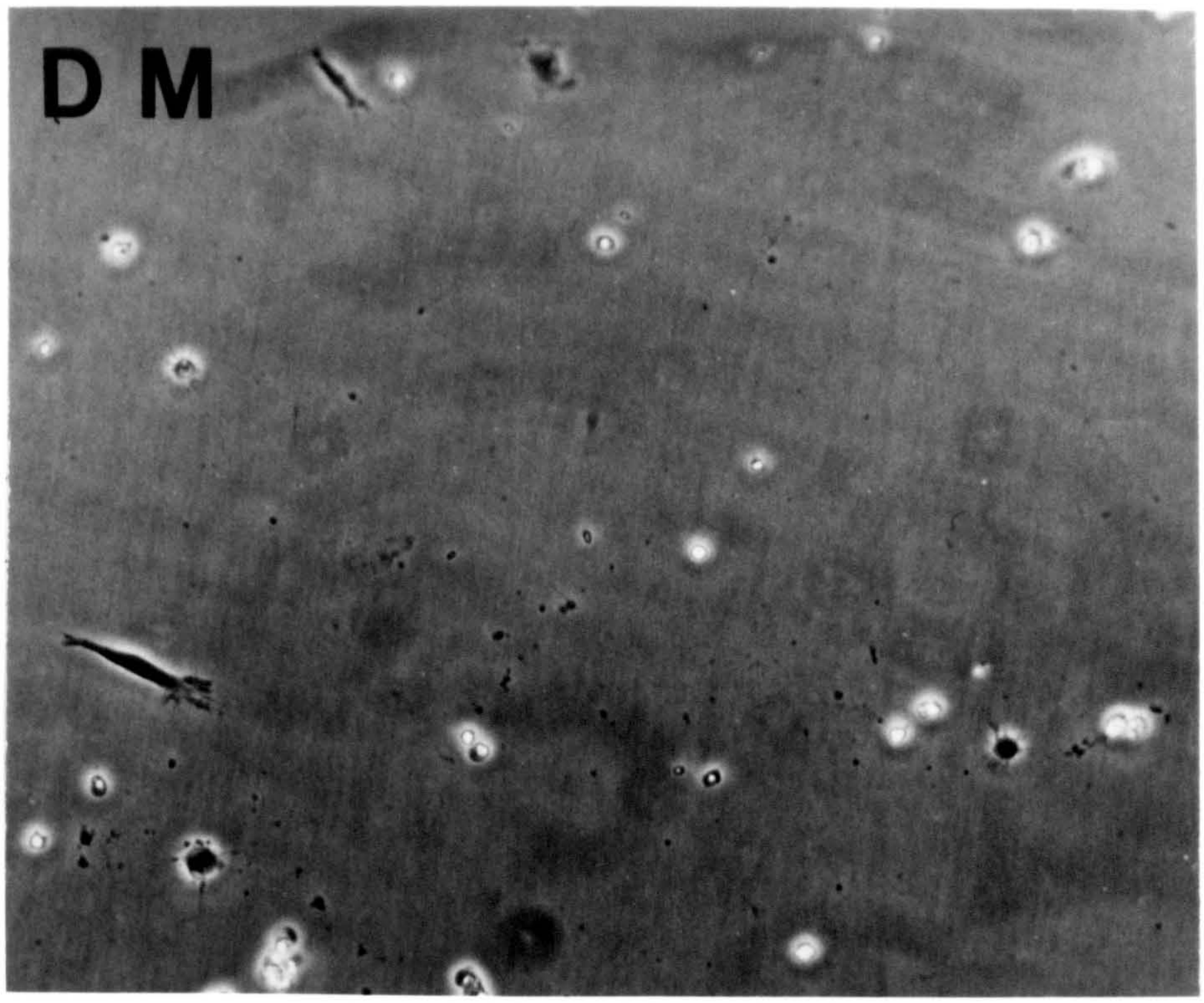

Constant bFGF 3ng/ml
IGF-1
IGF-2
INS
% SURVIVAL
120
100
80
60
40
20
0
0
.01
.1
1
10
100
1000
10K
CONCENTRATION ng/ml

Figure 5.2 Survival of Schwann cell precursors in the presence of bFGF and IGF

Phase micrographs of precursors after 20 hr in culture. (A) DM denotes culture in defined medium alone. (B) FGF + IGF denotes culture in the presence of 100ng/ml IGF-1 and 3ng/ml bFGF. In defined medium alone, most cells are dead after 20 hr; in the presence of bFGF and IGF-1, 100% of cells are alive at 20 hr post-plating. Bar = 50μm

A



B

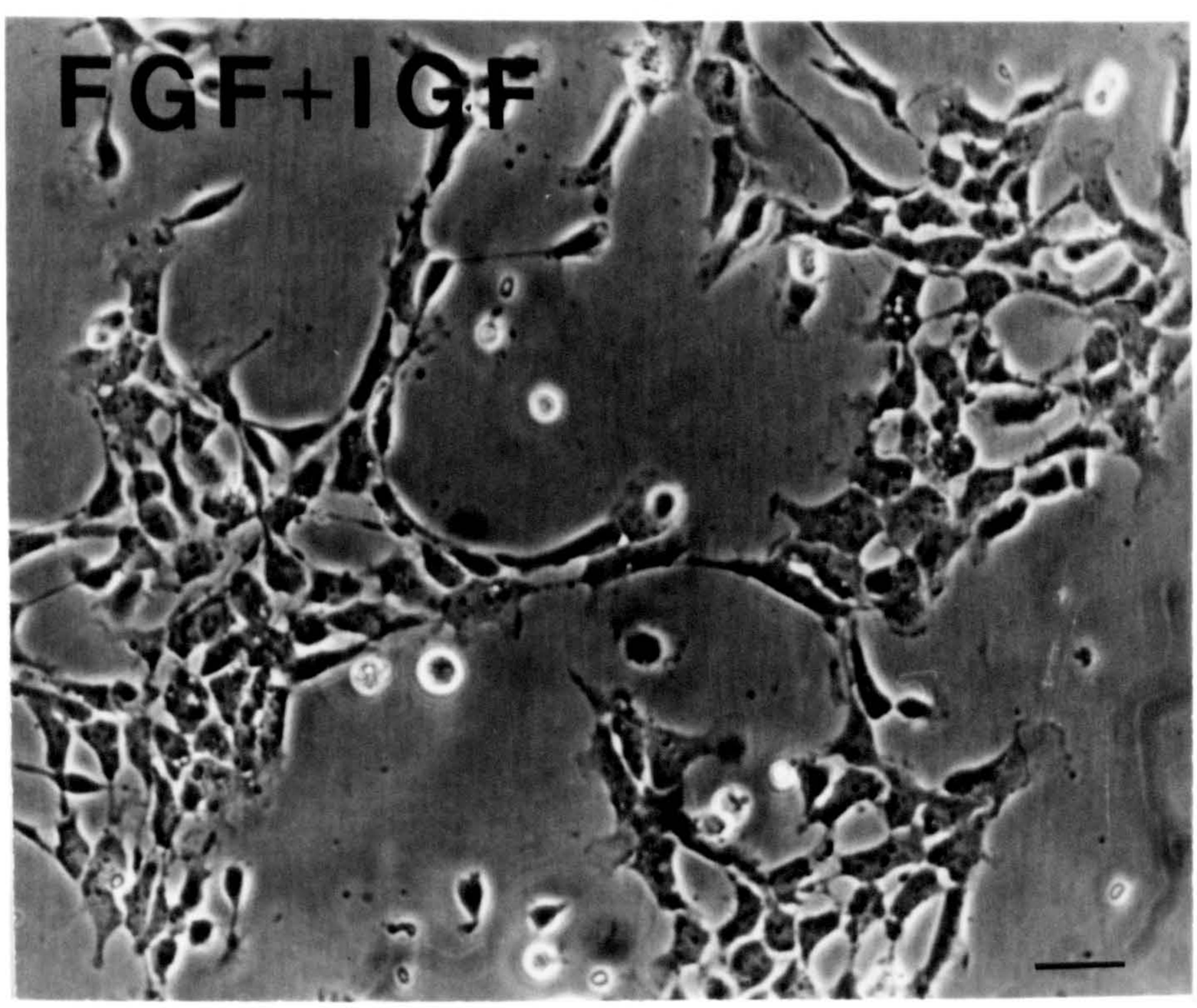


Figure 5.3 bFGF is required for IGF-1-mediated rescue of Schwann cell precursors

Cells were cultured in a constant concentration (100ng/ml) IGF-1 with increasing concentrations of bFGF. The response to bFGF showed a dose-dependent increase. Culture and immunolabelling is as described in the legend to Fig 5.1. The points represent an average of three experiments, error bars indicate SEM.

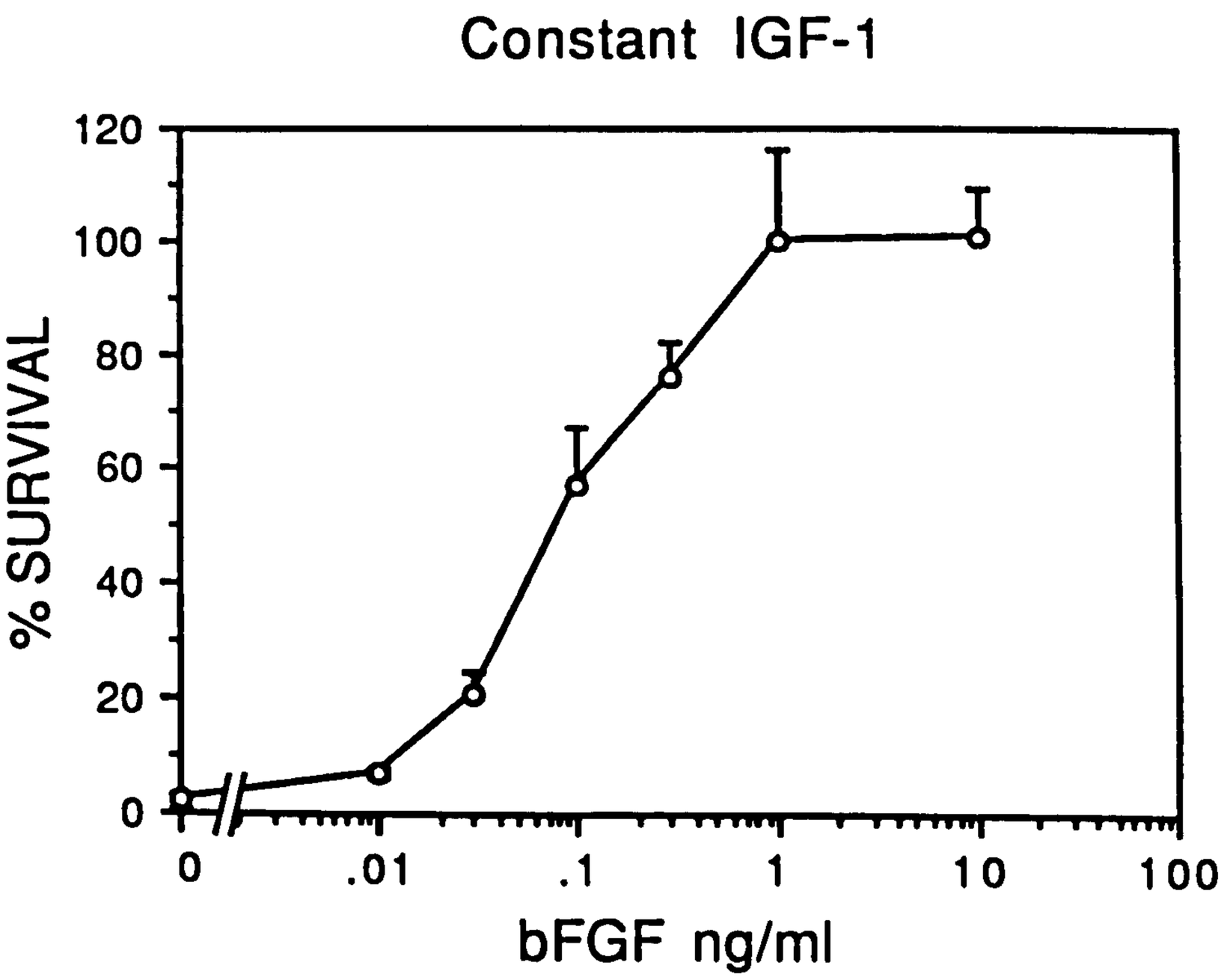
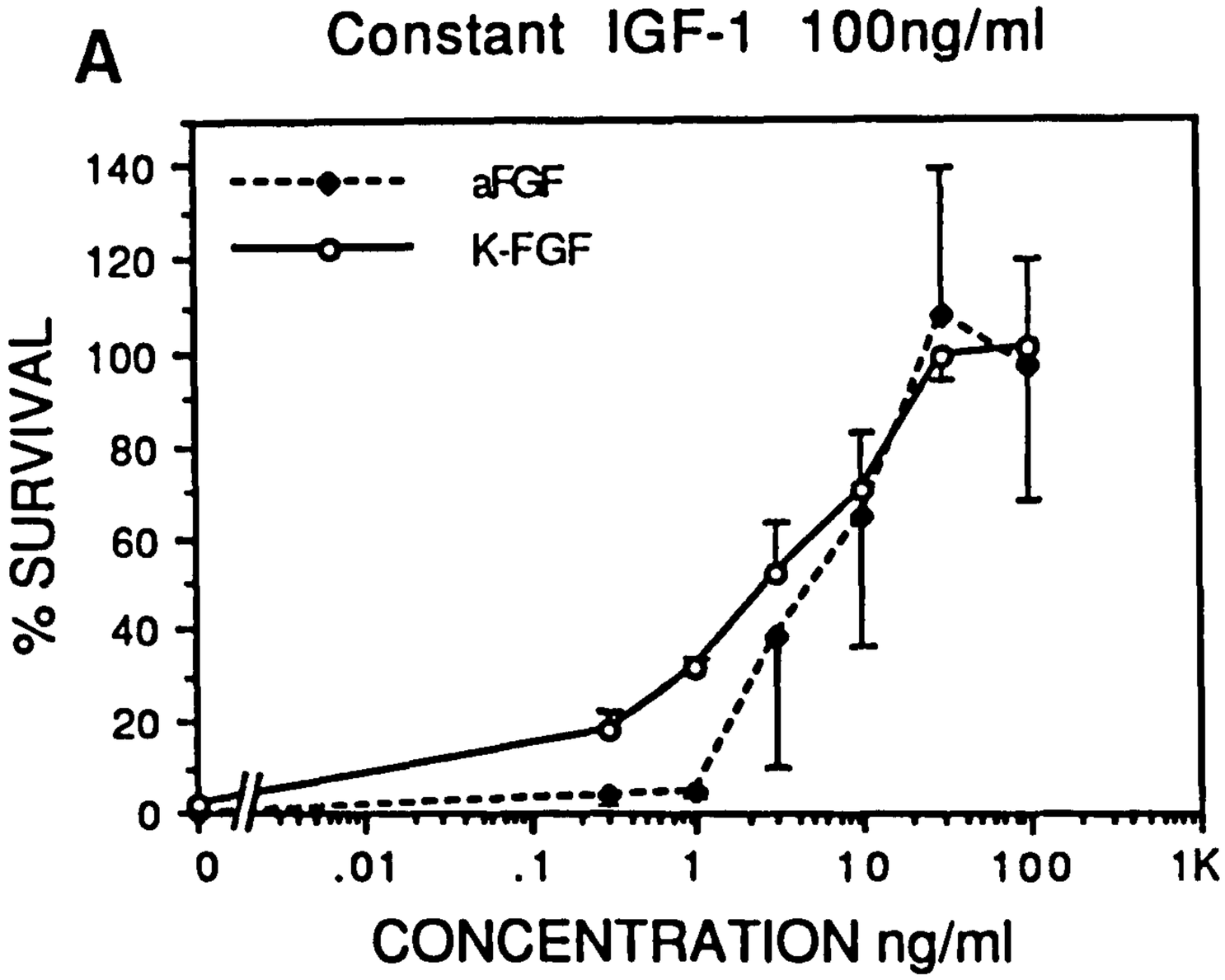

Constant IGF-1
% SURVIVAL
120
100
80
60
40
20
0
0
.01
.1
1
10
100
bFGF ng/ml

Figure 5.4 Effects of aFGF and K-FGF on Schwann cell precursor survival

Survival assays were performed as described in the legend to Fig 1, using increasing concentrations of aFGF or K-FGF in the presence of (A) a constant concentration of IGF-1 (100ng/ml), or (B) a constant concentration of insulin (1μM). (A) Both aFGF and K-FGF were capable of rescuing 100% of precursors in the presence of IGF-1. (B) High concentrations of insulin failed to mimic the survival effects of IGF-1. The points represent an average of three experiments, error bars indicate SEM.


A
Constant IGF-1 100ng/ml
aFGF
K-FGF
% SURVIVAL
140
120
100
80
60
40
20
0
0
.01
.1
1
10
100
1K
CONCENTRATION ng/ml

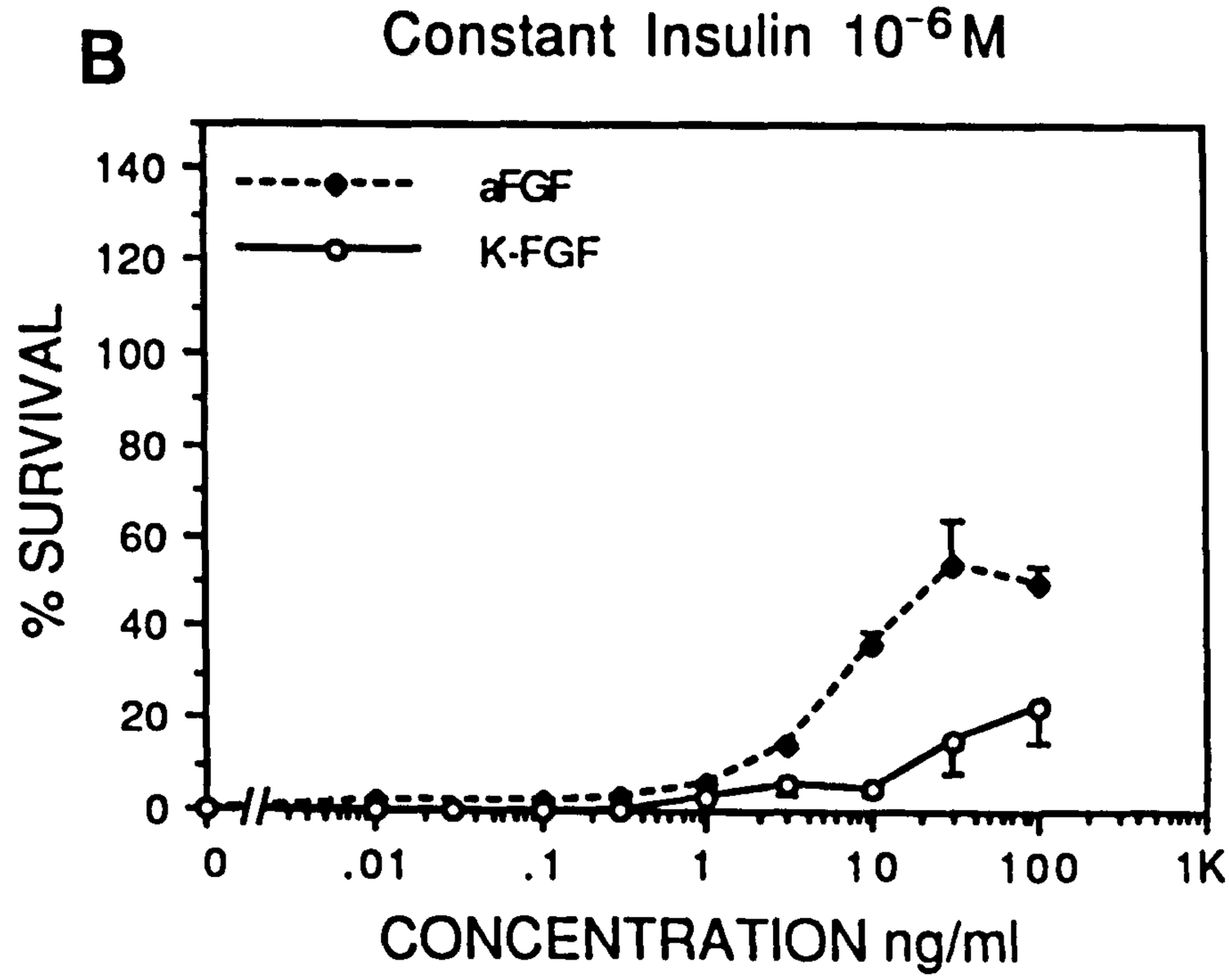

B
Constant Insulin 10^{-6}M
aFGF
K-FGF
% SURVIVAL
140
120
100
80
60
40
20
0
0
.01
.1
1
10
100
1K
CONCENTRATION ng/ml

Figure 5.5 Elevation of cAMP does not enhance survival of precursors in bFGF and IGF

Survival assays were performed both with and without the addition of 5μM forskolin. Constant concentrations of bFGF (3ng/ml) were used in the presence of increasing concentrations of (A) IGF-1 and (B) IGF-2. In (C), a constant IGF-1 concentration of 100ng/ml was used with increasing concentrations of bFGF. No significant enhancement of survival or shift in the dose-response curves was produced by elevation of cAMP. The points represent an average of three experiments, error bars indicate SEM.

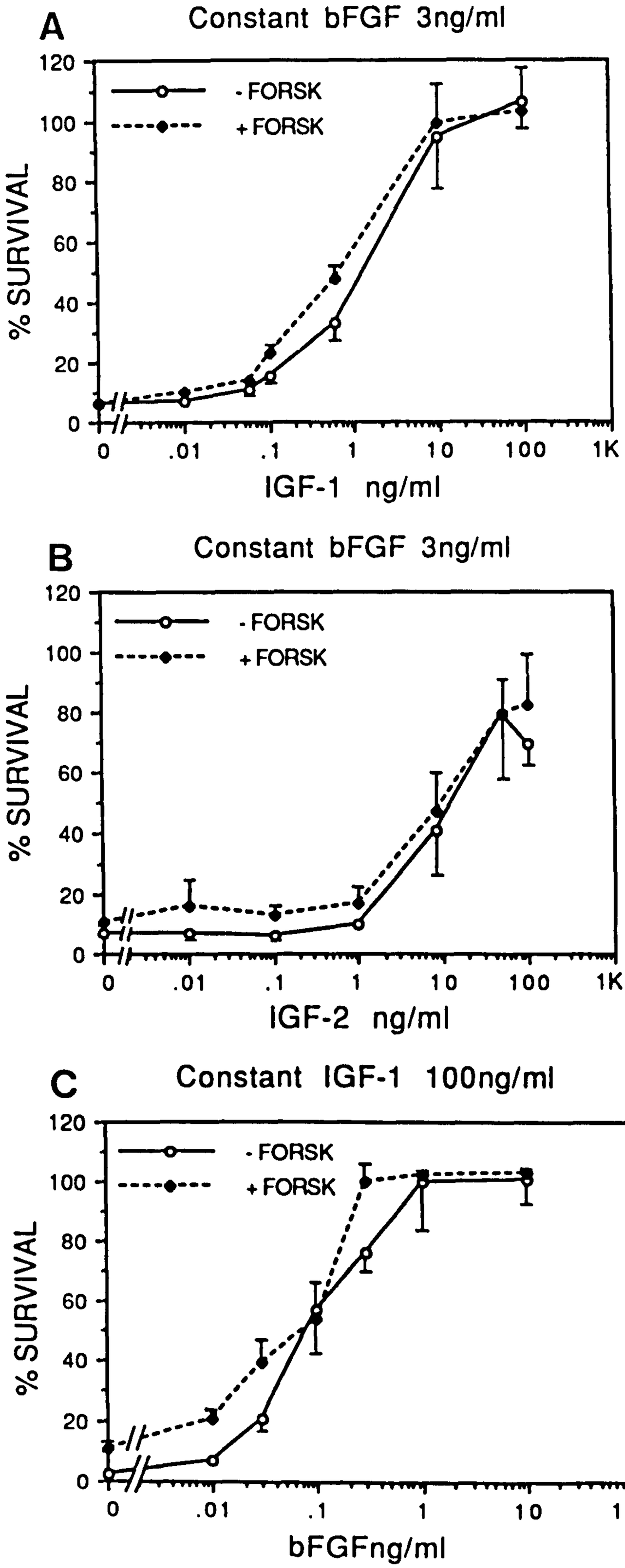
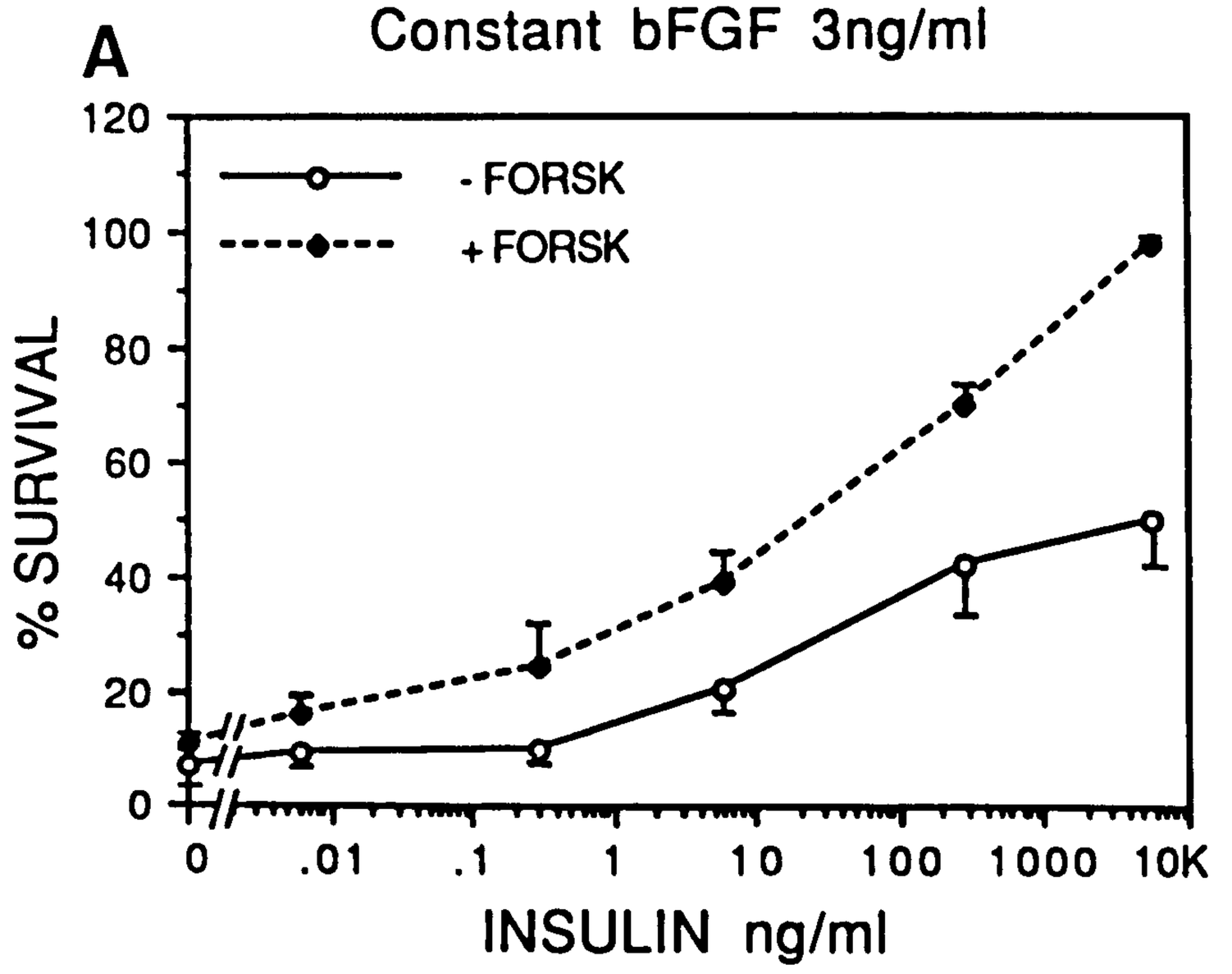

A
Constant bFGF 3ng/ml
- FORSK
+ FORSK
% SURVIVAL
120
100
80
60
40
20
0
0
.01
.1
1
10
100
1K
IGF-1 ng/ml
B
Constant bFGF 3ng/ml
- FORSK
+ FORSK
% SURVIVAL
120
100
80
60
40
20
0
0
.01
.1
1
10
100
1K
IGF-2 ng/ml
C
Constant IGF-1 100ng/ml
- FORSK
+ FORSK
% SURVIVAL
120
100
80
60
40
20
0
0
.01
.1
1
10
100
bFGFng/ml

Figure 5.6 Elevation of cAMP enhances survival of precursors in the presence of bFGF and insulin

Survival assays were performed both with and without 5μM forskolin, in the presence of (A) a constant concentration (3ng/ml) of bFGF with increasing concentrations of insulin, and (B) a constant concentration (1μM) of insulin with increasing concentrations of bFGF. In (A), forskolin enhanced the maximal survival level to 100% without altering the EC_{50} for insulin. In (B), in the presence of forskolin, the maximal survival level was increased to 100% and there was a 5-fold decrease in the EC_{50} value for bFGF. The points represent an average of four experiments in (A) and five experiments in (B), error bars indicate SEM.


A
Constant bFGF 3ng/ml
- FORSK
+ FORSK
% SURVIVAL
120
100
80
60
40
20
0
0
.01
.1
1
10
100
1000
10K
INSULIN ng/ml

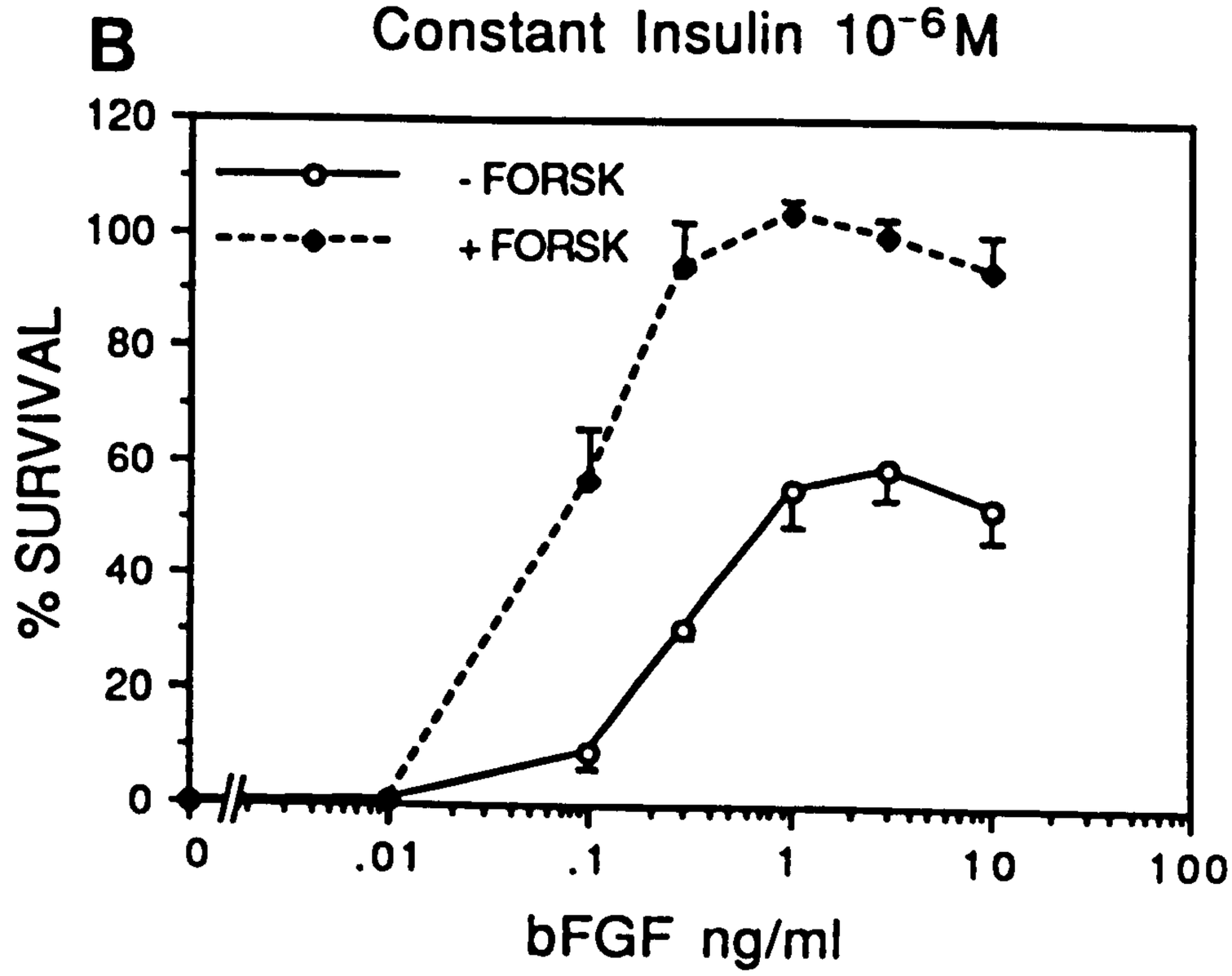

B
Constant Insulin 10^{-6}M
- FORSK
+ FORSK
% SURVIVAL
120
100
80
60
40
20
0
0
.01
.1
1
10
100
bFGF ng/ml

Figure 5.7 Elevation of cAMP promotes the survival effects of aFGF, K-FGF and K140 in the presence of insulin

Survival assays were performed both with and without 5μM forskolin, in the presence of a constant concentration (1μM) of insulin and increasing concentrations of (A) aFGF, (B) K-FGF, and (C) K-140. (A) The survival promotion by forskolin in the case of insulin/aFGF is similar to that seen with insulin/bFGF, with the EC_{50} lowered ~9-fold. (B) and (C) Forskolin produced a striking enhancement of survival, increasing the maximal survival in both factors from 20% to 100%. A decrease in the EC_{50} by ~5 fold in the case of K-FGF and by ~3-fold in the case of K-140 was also apparent. The points represent an average of three experiments, error bars indicate SEM.

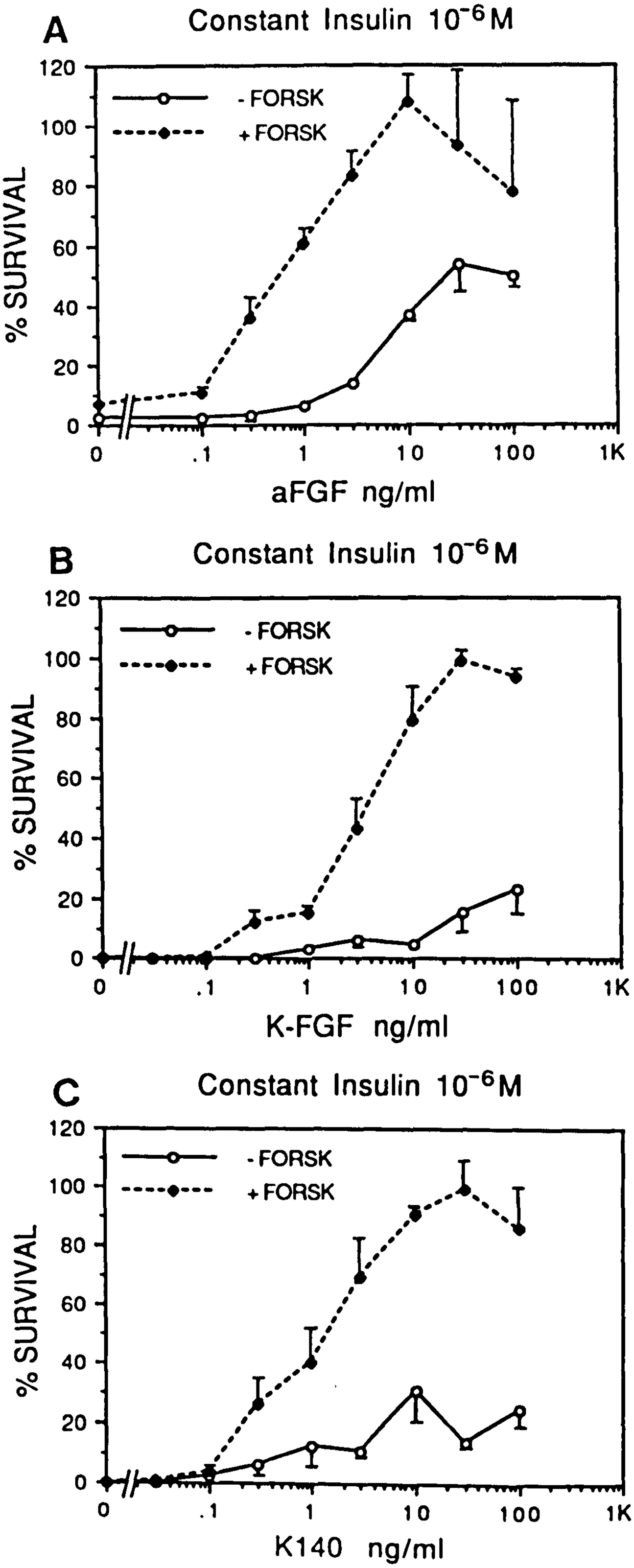

A
Constant Insulin 10^{-6}M
- FORSK
+ FORSK
% SURVIVAL
120
100
80
60
40
20
0
0
.1
1
10
100
1K
aFGF ng/ml
B
Constant Insulin 10^{-6}M
- FORSK
+ FORSK
% SURVIVAL
K-FGF ng/ml
C
Constant Insulin 10^{-6}M
- FORSK
+ FORSK
% SURVIVAL
K140 ng/ml

Figure 5.8 The effect of hormone omission from defined medium on precursor survival

The standard survival assay was performed in the presence of 100ng/ml IGF and 3ng/ml bFGF. The defined medium was deficient in one or all of the hormones present in the standard medium as indicated in the graph. Omission of the hormones did not significantly affect precursor survival (Students *t* test; $P < 0.5$), except for the omission of triiodothyronine (T3) where the difference was statistically significant (Students *t* test; $P < 0.01$). NORM: standard defined medium; -DEX: without dexamethasone; -PROG: without progesterone; -T3: without triiodothyronine; -T4: without thyroxine; -ALL: without dexamethasone, progesterone, triiodothyronine and thyroxine. The points represent an average of two experiments, error bars indicate SD.

Hatched bars: - forskolin; grey bars: + forskolin

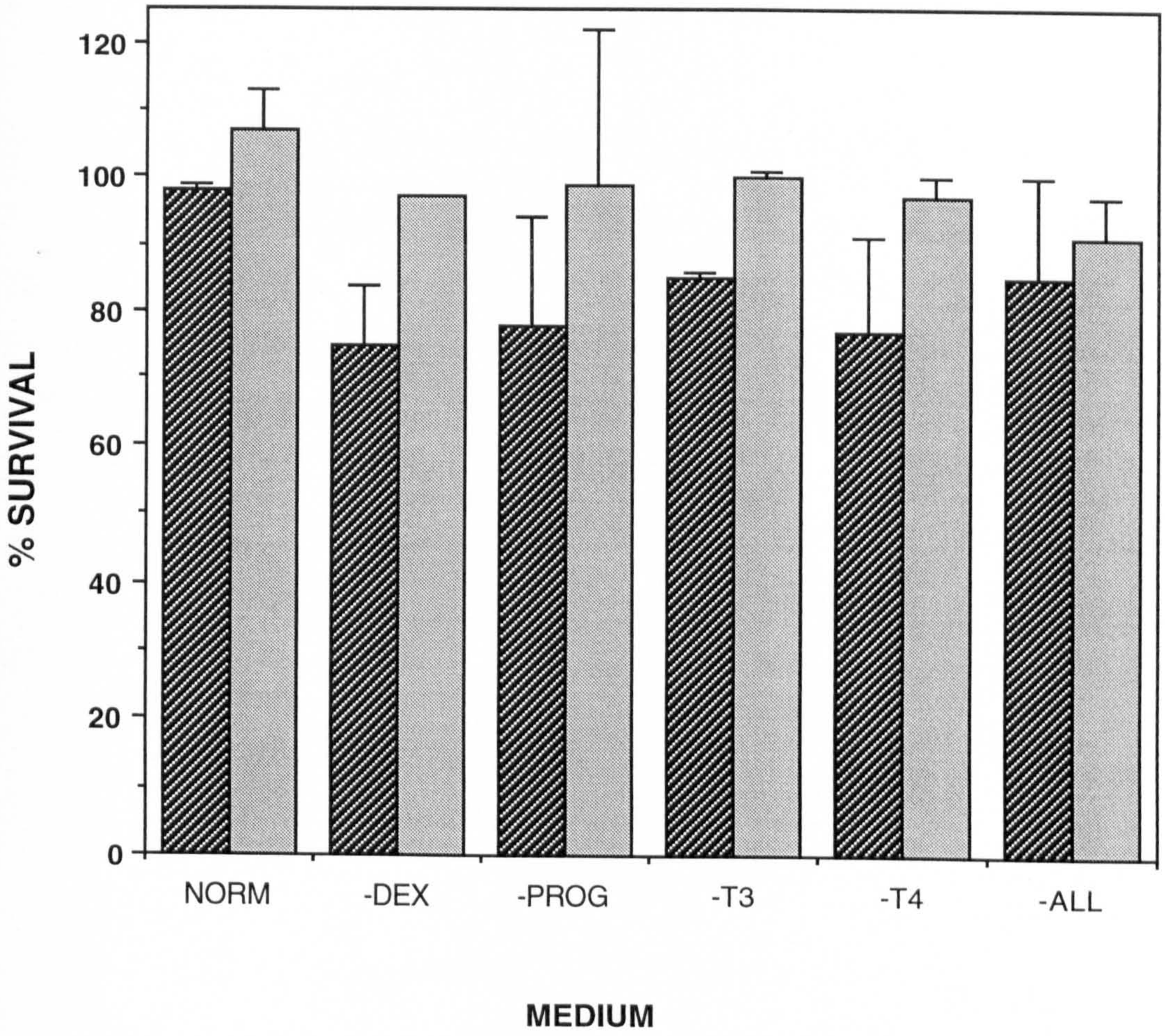

% SURVIVAL
120
100
80
60
40
20
0
NORM
-DEX
-PROG
-T3
-T4
-ALL
MEDIUM

Figure 5.9 The effect of substrates on precursor survival

A standard survival assay was performed in a constant concentration (1μM) of insulin with increasing concentrations of bFGF, both in the absence and presence (+ F) of 5μM forskolin. The dose-response curve for bFGF with cells on fibronectin-coated coverslips (solid bars) represents the average of 2 experiments, error bars indicate SD. The dose-response curve for bFGF with cells on laminin-coated coverslips (hatched bars) (already shown as Fig 5.6B) represent an average of 5 experiments, error bars indicate SEM. The survival curves were similar when different substrates were used, although fibronectin was less effective at the highest bFGF concentration, both in the absence and presence of forskolin.

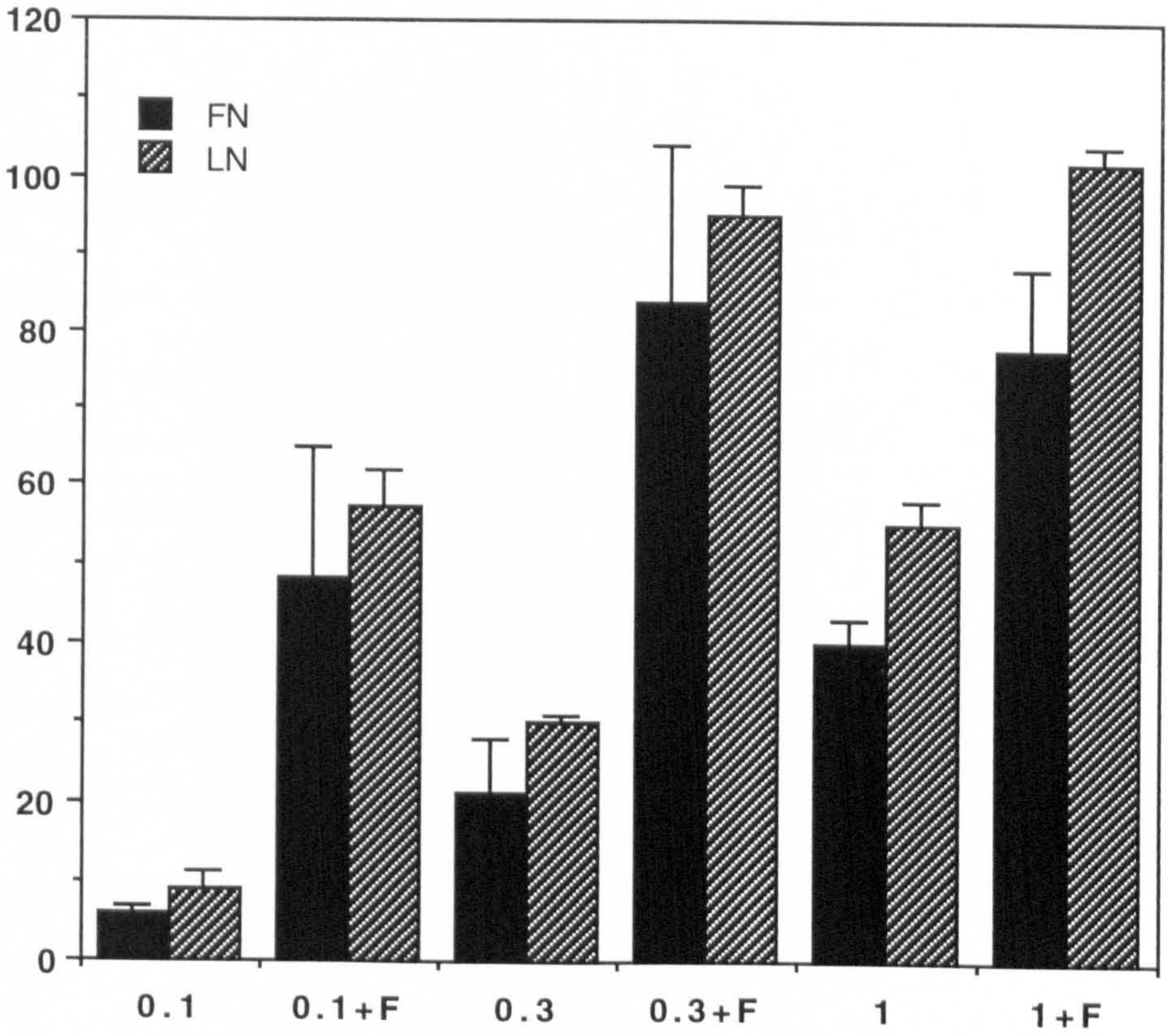
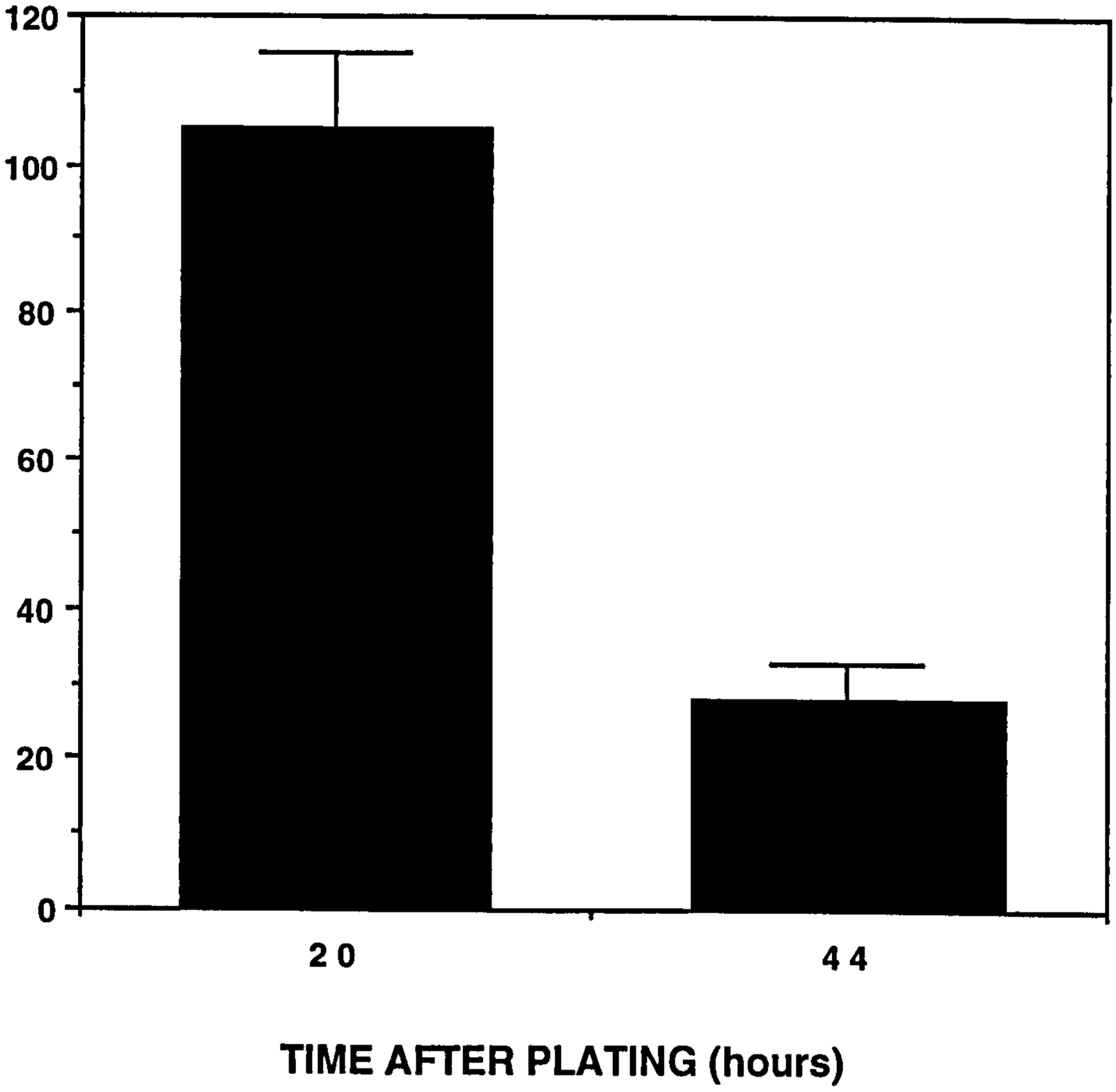

120
100
80
60
40
20
0
% SURVIVAL
FN
LN
0.1
0.1+F
0.3
0.3+F
1
1+F
bFGF CONCENTRATION (ng/ml)

Figure 5.10 bFGF does not promote the long term survival of precursors

Cells were cultured in 3ng/ml bFGF and 100ng/ml IGF-1 and immunolabelled for p75LNGFr at 20 hr or 44 hr after plating. The culture medium was replaced at 20 hr in the 44 hr cultures. The survival of the cells in this growth factor combination drops rapidly after 20 hr in culture. The points represent an average of two experiments, error bars indicate SD.


120
100
80
60
40
20
0
% SURVIVAL
20
44
TIME AFTER PLATING (hours)

DISCUSSION

Schwann cell precursors die by apoptosis when cultured in defined medium but will survive if the same medium has been conditioned by DRG neurones. This observation suggested that these cells require the presence of neurone-derived growth factor(s) for their survival in culture. Many growth factors were tested for their ability to support precursor survival in culture; three members of the fibroblast growth factor family, aFGF, bFGF and K-FGF, endothelins 1, 2 and 3, and neu differentiation factor (NDF) β2 were identified as potential survival factors in combination with an insulin growth factor. This Chapter has described a detailed analysis of survival conditions with FGFs and IGFs for the precursors *in vitro*.

Screening of candidate survival agents for E14 rat Schwann cell precursors

All the factors described in Table 5.1 and screened in the survival assay have been shown to affect either CNS glia, cells of the neural crest, or their derivatives, including Schwann cells. Despite many of the growth factors exhibiting survival effects on cells of other lineages, only the growth factors mentioned above were able to promote the survival of Schwann cell precursors.

Initial studies of all the factors were performed in medium that contained high levels (1μM) of insulin, a concentration that activates the type 1 IGF receptor (Rechler and Nissley, 1985). Later assays also included the addition of the cAMP-elevating agent forskolin, and the substitution of IGF-1 for insulin, as indicated in the table. Addition of forskolin or the presence of IGF did not enhance the effects of most of the factors that showed less than 10% survival in insulin alone. The addition of forskolin to high levels of insulin, and the substitution of IGF-1 for insulin, were effective at enhancing the survival effects of FGFs and will be discussed in detail below.

As described in Chapter 1, PDGF-AA and -BB, BDNF, NT-3, SCF and TGFβs are all present in neurones during development (Ernfors and Persson, 1991; Flanders et al., 1991; Hutchins and Jefferson, 1992; Schecterson and Bothwell, 1992; Langtimm-Sedlak et al., 1996). Similarly Schwann cells of perinatal and adult nerves have been shown to produce the above factors, with the exception of SCF (Scherer and Salzer, 1996). In addition, Schwann cells synthesize NGF, CNTF and LIF. Thus the growth factors are available in the nerve to act as paracrine or autocrine factors.

The lack of effect of all of the growth factors on the Schwann cell precursors was unexpected (Table 5.1). It is possible that, in some cases, these cells do not express functional receptors for the growth factor, or that the receptor number is low and occupancy cannot promote the survival of these cells. Weinmaster and Lemke (1990) have shown that elevation of cAMP in rat Schwann cells increases the number of PDGF β receptors. The time course of this increase is slow, however, with the increase in receptor mRNA first appearing after 24 hr. Even if elevating cAMP could increase receptor number in the precursors, the time course of death is so rapid that this could not promote the survival of these cells. Addition of forskolin to the precursor cultures with all the above growth factors showed no increase in survival.

The lack of effect of NGF on precursor survival is important since this was present in the NCM that produced precursor survival (Chapter 3; Jessen et al., 1994). These results show that the survival produced by NCM is not due to NGF alone.

α-melanocyte stimulating hormone has been shown to increase the number of melanocytes differentiating from quail neural crest (Satoh and Ide, 1987) and to induce melanogenesis from melanoblasts in newborn mouse skin (Hirobe and Takeuchi, 1977), via induction of adenyl cyclase (Abe et al., 1969; Hirobe and Takeuchi, 1977) but there is no evidence for survival factor activity in other systems.

The phorbol ester TPA activates protein kinase C (PKC) and thus can mimic the ligand-receptor binding effects of growth factors such as PDGF, FGFs and heregulins (Van der Geer, et al., 1994; Mochly-Rosen, 1995), as well as neurotransmitters and hormones that are activators of this enzyme. TPA has been reported to promote the survival of DRG neurones in culture (Montz et al., 1985) and enhances differentiation of oligodendrocytes in culture (Yong et al., 1988). The lack of effect of this agent on precursors would suggest that activation of PKC is insufficient to promote survival of these cells.

The Ca^{2+}-ionophore A23187 increases intracellular calcium by influx across the plasma membrane and by release of calcium from intracellular stores (McConkey and Orrenius, 1994). Ca^{2+} is a second messenger for many intracellular pathways, including those that involve 1,2-diacylglycerol (DAG) and PKC (Van der Geer et al., 1994), and has been implicated as an effector of apoptosis in thymocytes (Wyllie et al., 1984) and as an inhibitor of apoptosis as in the case of sympathetic neurones

(Edwards et al., 1991). Since in precursors, the developmental acquisition of survival in defined medium parallels the expression of the Ca^{2+}-binding protein, described in Chapter 3, the presence of nonphysiological concentrations of Ca^{2+} would be predicted not to promote the survival of the Schwann cell precursors and this is indeed the case.

These results indicate that survival of the precursors involves specific growth factor/ receptor interactions and that activation of PKC and increased intracellular Ca^{2+} cannot mimic the effect of the survival factor(s).

bFGF in combination with an insulin growth factor supports survival of E14 Schwann cell precursors

In these experiments, bFGF supports survival of precursors when added in the presence of either insulin, IGF-1 or IGF-2. As described above, bFGF has been reported to be a survival factor for chick nonneuronal crest cell derivatives *in vivo* (Kalcheim, 1989) and for chick and rat neural crest cells *in vitro* (Kalcheim, 1989; Bannerman and Pleasure, 1993). In this respect, the Schwann cell precursors have retained the growth factor requirement shown by cells at an earlier stage in development.

The level of survival observed with a given concentration of bFGF depends on the insulin growth factor present: the survival promoting effects of these factors are in the order IGF-1 > IGF-2 > insulin. These three factors are homologous, with IGF-1 and IGF-2 exhibiting an amino acid sequence homology of 62% to each other, and 43% and 41% homology, respectively, to proinsulin (Sara and Hall, 1990). Three receptors have been identified so far for these growth factors (reviewed in Neely et al., 1991). The insulin and the type 1 IGF receptors exhibit both structural and functional similarities. They are heterotetramers composed of disulphide-linked α and β subunits ($\alpha_2\beta_2$); the two α subunits form the extracellular ligand-binding domain, while the β subunits are transmembrane proteins with tyrosine kinase activity. The type 2 IGF receptor is also the cation-independent mannose-6-phosphate receptor with no homology to either the insulin or the type 1 IGF receptor; this single chain transmembrane protein does not have kinase activity but is thought to act via G-protein linkage (Humbel, 1990; Neely et al., 1991). These receptors exhibit variable binding for the growth factors: the insulin receptor binds insulin with high affinity, IGF-2 with lower affinity and IGF-1 with lowest affinity; the type-1 IGF receptor binds IGF-1 and

IGF-2 with high affinity and will also bind insulin at lower affinity, requiring the presence of high levels of insulin for activation (Rechler and Nissley, 1985; Sara and Carlsson-Skwirut, 1988); the type-2 IGF receptor binds IGF-2 with high affinity, IGF-1 with low affinity and does not bind insulin.

On the basis of these receptor affinities, it would appear that the receptor present on the Schwann cell precursors is likely to be the type 1 IGF receptor, since it responds to IGF-1, IGF-2 and high concentrations of insulin (1μM) (Rechler and Nissley, 1985; Sara and Carlsson-Skwirut, 1988; Neely et al., 1991). The mRNA for this receptor has been detected in Schwann cells *in vivo* and *in vitro* (Cheng et al., 1996) and the receptor is expressed on neonatal rat Schwann cells *in vitro* (Schumacher et al., 1993; Stewart et al., 1996b). The requirement for high insulin concentrations suggests that the survival effect is not mediated by the insulin receptor since the combination of bFGF with concentrations of insulin in the 5-50ng/ml range (~0.1-1.0nM) produced low levels of survival (Fig 5.1).

Both IGF and high concentrations of insulin can support the short-term survival of oligodendrocyte progenitors and oligodendrocytes *in vitro* without the presence of additional growth factors (Barres et al., 1992), and IGF-1 can support the survival of Purkinje neurones in culture (Torres-Aleman et al., 1992). IGF-1 and insulin block apoptosis induced by serum deprivation in Rat-1 fibroblasts and COS-1 cells transfected with ICE by preventing processing and subsequent activation of this protease (Jung et al., 1996). However, the presence of both an FGF and an IGF or insulin is required for Schwann cell precursor survival (Figs 5.1, 5.3, 5.4), suggesting either that the activation of IGF receptors is not sufficient to promote the survival of these cells, or that FGFs modulate the type-1 IGF receptors as has been shown in other cell types. Purified glial cells from hypothalamic cultures express increased numbers of type-1 IGF receptors in response to bFGF (Pons and Torres-Aleman, 1992); similarly, bFGF increases the number of IGF receptors expressed by cultured vascular smooth muscle cells (Ververis et al., 1993). It is possible, therefore, that the presence of bFGF upregulates the number of type-1 IGF receptors on the Schwann cell precursors and thus enhances the response to IGF-1, IGF-2 or insulin and thereby promotes survival. This action would have to be rapid, however, since the growth

factors are added to the precursor cultures at 3 hr post-plating and the precursors begin to die within 5 hr post-plating if the correct growth factors are not present.

The actions of IGFs are modulated by binding proteins; six IGF binding proteins (IGFBP) have been described so far. These binding proteins can enhance or inhibit IGF activity, possibly by affecting the binding of IGF to receptors (Elgin et al., 1987; Sara and Hall, 1990; Lamson et al., 1991). Schwann cells have been shown to express IGFBP-5 and another IGFBP with a molecular weight consistent with either IGFBP- 4 or -6 (Cheng et al., 1996). The level of IGFBP-5 is upregulated in these cells by the action of IGF-1 which appears to protect the binding protein from proteolysis. It has been shown that bFGF can modulate the synthesis of one of the IGFBPs in cultures of purified hypothalamic neural cell cultures (Ernst and Rodan, 1990; Pons and Torres-Aleman, 1992) and of IGFBPs 2 and 3 in cultures of rat astrocytes (Loret et al., 1991). This represents another mechanism by which FGFs could affect the response of the Schwann cell precursors to IGFs, but, as with the possible upregulation of IGF receptors, this effect would have to be rapid.

As described earlier, high concentrations of insulin activate the type-1 IGF receptor (Sara and Hall, 1990) and in other systems such as cultured rat sympathetic neuroblasts (DiCicco-Bloom and Black, 1988) and rat oligodendrocytes (Barres et al., 1992) both IGF-1 and high concentrations of insulin produce similar responses. In the system described here, however, the synergy of insulin and IGF with bFGF are not interchangeable (Fig 5.1). The presence of bFGF and IGF-1 promoted the survival of 100% of precursors at 20 hr, but at 1μM insulin the survival in bFGF was maximally 50%. When the insulin concentration was increased to 4μM the survival in the presence of bFGF actually dropped by 10%, suggesting that the difference in effect was not due to a sub-optimal concentration of insulin. It is possible that the survival response may be enhanced by signalling via type 2 IGF receptors that do not bind insulin: these receptors are expressed by Schwann cells in culture (Stewart et al., 1996b). However, it is not clear whether the type 2 receptor has a role in mediating effects of IGFs in other systems (Neely et al., 1991). The modulating actions of IGFBPs may also be involved in the IGF/insulin differences; these have been shown to bind and enhance the mitogenic effect of IGF-1 on fibroblasts, but do not affect the response of these cells to insulin (Elgin et al., 1987; Conover et al., 1990).

Alternatively, IGF-1 may protect the IGFBPs from rapid proteolysis (Cheng et al., 1996), an effect that may not be mimicked by insulin if it does not bind efficiently to IGFBPs.

Four hormones are present in the standard defined medium used in these experiments, dexamethasone, progesterone, thyroxine and triiodothyronine. Oligodendrocyte progenitors have been reported to respond to progesterone by increased survival (Barres et al., 1993) and both dexamethasone and triiodothyronine can influence the timing of oligodendrocyte differentiation from precursors (Barres et al., 1994b). In Schwann cells, progesterone has been reported to increase myelin sheath formation after lesion and can be synthesized by these cells (Koenig et al., 1995). Omission of the hormones from the precursor culture medium in the presence of bFGF and IGF, in turn or all together, did not result in a dramatic decrease in precursor survival. However, omission of triiodothyronine gave a statistically significant decrease in cell survival but this effect was lost when all four hormones were omitted (Fig 5.8). This may reflect the requirement for triiodothyronine in the presence of the other hormones, whose effects may be deleterious for precursor survival in its absence.

Acidic FGF and K-FGF also synergize with an IGF to promote precursor survival

Both aFGF and K-FGF were able to promote the survival of the precursors in combination with an insulin growth factor. The effect of K-FGF as a survival agent is important since this is a secreted form of FGF, while aFGF and bFGF do not possess conventional signal sequences (Delli-Bovi et al., 1988; Basilico and Moscatelli, 1992). As with bFGF, combination of these factors with IGF produced 100% survival, while combination with insulin rescued far fewer cells (Fig 5.4 B). Acidic FGF promoted similar survival to bFGF under these conditions (Figs 5.1, 5.4 B) while K-FGF was able to rescue only 20% of the cells. The truncated K-FGF molecule, K140, that has been reported to have five times higher biological activity than K-FGF (Bellosta et al., 1993), had a similar pattern of survival to K-FGF in insulin (Fig 5.7 C), and in line with higher biological activity, the EC_{50} in insulin showed a three-fold decrease in concentration compared to K-FGF.

In other experiments, it has been shown that FGF-5, and *in vitro* translates of int-2 (FGF-3) and KGF (FGF-7) do not have survival activity in this assay (J. Gavrilovic,

M. Mattieu and K. R. Jessen, unpublished data) suggesting a selective response to the FGF receptors present on the Schwann cell precursors. As mentioned above, the different members of the FGF family bind to the four mammalian high affinity FGF receptors with varying affinities. Since aFGF, bFGF, and K-FGF bind to FGFR-2 with similar affinities, it is a good candidate for the receptor responsible for the survival signal in these cells.

cAMP enhances the survival effects of FGFs in insulin

The addition of forskolin to cultures of precursors in the presence of FGFs and insulin increased the total number of cells surviving at 20 hr and shifted the dose-response curves to the left, indicating an increase in receptor affinity (Figs 5.5, 5.6, 5.7). In the presence of IGF-1 or IGF-2, however, there was no significant change in either the cell survival or receptor affinity in the presence of bFGF. If forskolin increased the binding affinity of FGF for the receptor, there should be some difference in the EC_{50} seen in bFGF/IGF. This was not the case, however, suggesting that the enhanced survival is a result of an increase in affinity of insulin for the IGF type-1 receptor (Fig 5.6 A, B). The increase in the number of cells surviving in the presence of insulin/FGF and forskolin may be due to recruitment of cells previously unresponsive to insulin. This could occur either by upregulation of IGF receptor expression by cAMP, or by the enhancement of intracellular signals generated in response to insulin/type-1 receptor binding. Synergy between cAMP elevation and insulin action has been reported in fibroblasts, enhancing mitogenesis (Rozengurt et al., 1981), and forskolin has been reported to increase the number of type-1 IGF receptors on Schwann cells in culture (Schumacher et al., 1993). This increase in receptor number, however, was observed after 3 days exposure to the factors. Such an effect may be relevant *in vivo*, where cAMP-elevating signals and IGF may play a role in precursor survival, but, as described earlier, receptor synthesis *de novo* in this *in vitro* system would hardly be rapid enough to influence the survival of these cells.

FGFs and IGFs are not mitogenic for precursors

Both IGFs and FGFs are well known mitogens for many cell types (reviewed in Sara and Hall, 1990; Basilico and Moscatelli, 1992). This combination of growth factors, in the presence of elevated cAMP, is mitogenic for Schwann cells in culture (Stewart et al., 1991; Schumacher et al., 1993). The precursors, however, failed to divide in

response to these factors indicating that the regulation of DNA synthesis differs between these cells and Schwann cells. Thus the response of cells to this combination of growth factors is developmentally controlled. Work by Z. Dong (Dong et al., 1995) has shown that the cells develop the ability to proliferate in response to this growth factor combination with a time course that parallels the acquisition of survival in defined medium, and in Chapter 6, this response is used as one of the distinguishing features of the precursor to Schwann cell transition.

The effect substrate on precursor survival

The substrate used for the culture conditions appeared to play an important role in precursor survival. All the experiments described here were performed on laminin coated coverslips, but identical experiments on PLL coated coverslips resulted in the survival of no more than 10% of the starting population. In contrast, fibronectin coated coverslips promoted similar survival to laminin at low concentrations of bFGF (Fig 5.9). However, in both experiments on fibronectin, a concentration of bFGF (1ng/ml) that produced maximal survival response on laminin, was slightly less effective on fibronectin; further experiments would reveal whether this is a real phenomenon.

The solutions of both laminin and fibronectin used are relatively crude preparations which may contain other growth factors. It is possible that the survival effects observed with IGFs and FGFs require these additional factors for their affect. However, several batches of laminin were used during the course of these experiments and no differences in survival were observed. The lack of survival in PLL may be due to cytotoxic effects of this compound that are negated by coating these coverslips with laminin or fibronectin. Alternatively, it may be because the precursors require the presence of extracellular matrix molecules for survival, since the PLL coverslips do not kill neonatal Schwann cells. Both laminin and fibronectin are found along the path of migrating neural crest cells (reviewed in Bronner-Fraser, 1993) and laminin is associated with the earliest axons growing into the periphery in the chick (Rogers et al., 1986). Integrin receptors for extracellular matrix molecules have been described on both neural crest cells (reviewed in Bronner-Fraser, 1993) and on Schwann cells (Lefcort et al., 1992; Niessen et al., 1994). Addition of bFGF to cultures of chick neural tube cells promotes the adhesion of these cells to collagen, laminin or

fibronectin substrates by upregulating β_1 integrins (Kinoshita et al., 1993). Moreover, clustering of integrins can induce intracellular changes such as protein phosphorylation (Kornberg et al., 1991). Embryonic chick ciliary ganglion neurones require the binding of laminin to the integrin receptor β1 for bFGF to promote the survival of these neurones (Schmidt and Kater, 1995). Therefore attachment of the precursors via integrins may be important in promoting their survival in conjunction with exogenous growth factors.

FGFs and IGFs are present in the developing nerve

For these factors to influence the development of the Schwann cell precursors they must be present in the PNS at an appropriate time. Acidic FGF has been reported to be absent from E15 DRG and is first detected at both the mRNA and protein levels in neurones of the E18 rat (Oellig et al., 1995), but in the same study S100 protein is not found until E21, some 4-5 days after it is first detectable by immunocytochemistry as described in Chapter 3. The earliest available data for the expression of bFGF *in vivo* is that of the E18 rat where it is detectable by immunocytochemistry in DRG neurons (Gonzalez et al., 1990). Both aFGF and bFGF are present in DRG neurones isolated from E15 rat and maintained in culture (Neuberger and De Vries, 1993). In the chick, mRNAs for aFGF and bFGF protein are expressed from E6 onward (approximately equivalent to E14 rat) in spinal cord and DRG neurones (Kalcheim and Neufeld, 1990; Schnurch and Risau, 1991). Acidic FGF is found at high levels in the adult rat sciatic nerve, while bFGF is not detected (Eckenstein et al., 1991). The distribution of K-FGF in the developing peripheral nervous system has yet to be documented, but mRNA for K-FGF has been found at early stages in mouse development (Hebert et al., 1990).

As yet there is no direct localization of any of the FGF receptors to Schwann cells or their precursors. The mRNA for FGFR1 has been detected in the rat DRG at E14 (Wanaka et al., 1991). Oellig et al. (1995) report that mRNA for both FGFR1 and FGFR2 are associated with DRG neurones from E15 in the rat, but that the glial cells are negative; FGFR3 and FGFR4 are absent from the developing DRG. The radioactive detection method used in this study shows a diffuse signal throughout the early DRG and it is impossible to say with certainty that the glial cells do not possess FGFRs.

IGF-1 and IGF-2 mRNA are expressed throughout the developing rat embryo, and at E15 are found in undifferentiated mesenchyme of the developing limb, around the spinal ganglia and sympathetic trunk (Bondy et al., 1990). These studies also show IGF-1 mRNA in mesenchymal cells condensing around the developing nerve at E15. Strong IGF immunoreactivity has been detected within chick peripheral nerves (Ralphs et al., 1990) and immunoreactivity for IGF-1 has been found in Schwann cells of adult rat sciatic nerve (Cheng et al., 1996).

The distribution of IGF receptors on cells of the developing nervous system have not been extensively studied. Bondy et al, (1990) have found type-1 IGF receptor mRNA throughout the embryonic nervous system and associated with the DRG. As described earlier, type-1 receptor mRNA has been detected in adult rat Schwann cells *in vivo* (Cheng et al., 1996). Schwann cells from neonatal nerves possess type-1 receptors as inferred from radiolabelled IGF-1 binding studies; non-radioactive IGF-1 was most effective at blocking radiolabelled IGF-1 binding, and 1μM insulin was only partially effective (Stewart et al., 1996b). Type-2 receptors are also present on these neonatal Schwann cells and in fact are expressed at higher levels in Schwann cell precursors (Stewart et al., 1996b; H. J. S. Stewart, personal communication).

The distribution of FGFs and IGFs suggest that these factors are spatially and temporally available in the developing embryo for trophic support of the Schwann cell precursors.

The effect of FGFs and IGFs on precursor survival is only short term

Despite promoting the survival of 100% of the precursors in a 20 hr assay, bFGF and IGF or bFGF, insulin and forskolin cannot support long term survival of these cells. Only 28% of the cells that have attached at 3 hr survive culture under these conditions for 44 hr. The short-term survival effects of aFGF have been reported in PC12 cells, although 'short-term' is relative, since the survival in this case is two weeks in culture, compared to survival produced by NGF of months (Claude et al., 1988). Oligodendrocytes require the presence of three growth factors, an IGF, a neurotrophin and a neurokine for their long term survival; the presence of a single factor from each of these families promotes only short-term (3d) survival (Barres et al., 1993).

Both FGF and IGF receptors are internalized and therefore downregulated after ligand-binding (Sara and Hall; 1990; Walicke and Baird, 1991). Such downregulation may make the cells unable to respond to the survival factors after prolonged exposure. The precursors have not lost the ability to survive in culture, since changing the medium at 20 hr from one containing IGF/bFGF to one containing NDFβ-2 will allow all the cells alive at 20 hr to survive indefinitely (Dong et al., 1995). Since the evidence from the experiments described here suggest that IGF receptor binding is critical in promoting the survival of the precursors, down regulation of this receptor would render cells unable to respond to the factor. It is interesting to note that NDFβ-2 is able to promote precursor survival in the absence of insulin or IGF-1 (Dong et al., 1995), and thus could effect a response even under conditions where IGF receptors are down regulated.

In conclusion, three members of the FGF family, aFGF, bFGF and K-FGF, can synergize with a member of the IGF family of growth factors to promote the short-term survival of Schwann cell precursors. The presence of a member from both of the families is required for survival. The effects of an FGF and insulin are enhanced by elevation of cAMP, but the substitution of an IGF for insulin produces maximal survival that is not significantly altered by cAMP elevation. The addition of these factors does not result in DNA synthesis.

CHAPTER 6

LONG TERM SURVIVAL AND MATURATION OF SCHWANN CELL PRECURSORS

INTRODUCTION

In the previous chapter, many factors were tested for their ability to promote the survival of the Schwann cell precursors. The effects of the FGFs have been described, but members of two other families of growth factors also supported survival in culture: NDFβ-2, a member of the neuregulin family of growth factors, and endothelins 1, 2 and 3.

Neu differentiation factor

The neu differentiation factors (NDFs) are members of the neuregulin family of growth factors which are alternatively-spliced products of a single gene (Ben-Baruch and Yarden, 1994). Members of the family have been cloned from different species, hence the variety of names given to the factors in the group: NDF was cloned from rat (Wen et al., 1992), heregulin from human (Holmes et al., 1992), acetylcholine receptor-inducing activity (ARIA) from chick (Falls et al., 1993), and glial growth factor was cloned from bovine brain (Marchionni et al., 1993); at least 15 different variants are known, derived from mesenchymal and neuronal tissue (Peles and Yarden, 1993; Burgess et al., 1995).

The precursors of these factors are membrane-associated polypeptides with isoforms based around a structure comprising a variable N-terminal domain, an Ig motif, a spacer domain with glycosylation sites, an EGF-like domain, an hydrophobic transmembrane region and a variable cytoplasmic tail (Wen et al., 1992; Peles and Yarden, 1993; Ben-Baruch and Yarden, 1994). The isoforms are designated α or β depending on the sequence of the third loop in the EGF-like domain. Variation in the extracellular juxtamembrane region produces the subtypes 1-5 (Ben-Baruch and Yarden, 1994). Most NDF isoforms can be secreted, being cleaved by proteolysis in the juxtamembrane domain, the exception being NDFβ-3 which lacks the transmembrane domain (Ben-Baruch and Yarden, 1994; Burgess et al., 1995); the secreted rat NDFs have molecular weights of ~ 40-44kD. However, NDFs are also found in membrane-bound form which may be important in direct cell-cell signalling *in vivo* (Burgess et al., 1995). NDFs are produced by both mesenchymal and neuronal tissues, with pro-NDFα-2 as the predominant form in the mesenchyme and pro-NDFβ-1 as the major isoform in neuronal tissue (Wen et al., 1994). The presence of a

kringle-like domain at the N-terminus is found only in neuronally-expressed NDFs (Ben-Baruch and Yarden, 1994).

Three receptors have so far been identified for NDF: Neu/HER-2/ErbB-2, HER-3/ErbB-3 and HER-4/ErbB-4 (Bargmann et al., 1986; Carraway et al., 1994; Tzahar et al., 1994; Wen et al., 1994). They are protein tyrosine kinases of the EGF-receptor family (Peles and Yarden, 1993) that on binding ligand form dimers (Marchionni, 1995). The interaction of NDFs with these receptors and the subsequent intracellular signalling is complex. Constitutive expression of ErbB-2 by human ovary tumour cell lines or transfection of fibroblast cell lines with ErbB-2 cDNA does not confer high affinity binding for NDF on these cells and there is no phosphorylation of the receptor in the presence of ligand (Peles et al., 1993). Transfection of a bovine ErbB-3 cDNA into insect cells generated a receptor with a low affinity for NDF; in these cells and in fibroblasts transfected with ErbB-3 cDNA, the presence of ligand receptor resulted in tyrosine phosphorylation of the receptor (Carraway et al., 1994). ErbB-4 exhibits higher affinity for NDFs than ErbB -3 and ligand binding also produces receptor phosphorylation (Plowman et al., 1993a). The presence of ErbB-2 and ErbB-3 together results in a high affinity binding site for NDFs with both receptor proteins being phosphorylated after binding ligand (Sliwkowski et al., 1994; Tzahar et al., 1994) and similarly, co-expression of ErbB-2 and ErbB-4 results in phophorylation of both receptors in response to NDF (Plowman et al., 1993b). None of the NDF isoforms activate the EGF-R/ErbB-1 receptor (Peles and Yarden, 1993) but NDF has been reported to inhibit EGF binding to its receptor by interference with heterodimerization of ErbB-1 with other ErbB proteins (Karunagaran et al., 1995).

As described above, following ligand binding and receptor dimerization, the receptors undergo autophosphorylation on 5 tyrosine residues that are not part of the kinase domain. These residues act as docking sites for proteins that contain the *src*-homology-2 domain that include phospholipase Cγ, phosphatidylinositol 3' kinase, and the GTPase activating protein of *ras*. Activation of the ErbB receptors by NDFs results in an increase in intracellular calcium, increased lipid turnover, increased glucose transport and rapid induction of c-fos and c-jun (Ben-Baruch and Yarden, 1994).

The β isoforms of NDF are active in both survival and mitogenesis of Schwann cells and Schwann cell precursors while the α isoforms are ineffective (Dong et al., 1995). NDFs are survival factors for other cells of neural origin, affecting cells of the cranial ganglia *in vivo* (Meyer and Birchmeier, 1995), astrocyte survival *in vitro* (Pinkas-Kramarski et al., 1994) and teloglia at developing neuromuscular junctions (Trachtenberg and Thompson, 1996).

Endothelins

The endothelins (ETs) were originally identified as factors released by endothelial cells causing prolonged vasoconstriction (Yanagisawa et al., 1988). They are small peptide growth factors of 21 amino acids, ~MW 2.5 kD, with very similar amino acid sequences, ET-2 differs from ET-1 in 2 amino acids and ET-3 differs from ET-1 in 6 amino acids (Ohlstein and Ruffolo, 1995). They form identical structures with two intrachain disulphide bonds, an identical 6 amino acid C-terminal sequence (Yanagisawa et al., 1988; Ohlstein and Ruffolo, 1995) and are structurally similar to the snake venom toxins - the sarafotoxins (Yanagisawa and Masaki, 1989).

Each ET is encoded by a separate gene and is synthesized as a precursor protein of ~200 amino acids (Yanagisawa et al., 1988; Simonson and Dunn, 1991). The precursor protein, prepro-ET, is cleaved by dibasic-pair-specific endopeptidases (Yanagisawa et al., 1988) to yield pro-ET, also known as big-ET (Yanagisawa et al., 1988; Simonson and Dunn, 1991). This precursor peptide is then cleaved to give mature ET by a neutral metalloprotease, the endothelin converting enzyme, although other enzymes such as serine proteases may also be able to generate mature ET from the precursor protein (Ohlstein and Ruffolo, 1995).

To date, two main subtypes of endothelin receptor have been described, ET_A and ET_B. ET_A receptors bind ET-1 and ET-2 with high affinity and ET-3 with low affinity, while ET_B receptors are non-isopeptide selective, binding all three peptides with equal affinity (reviewed in Miller et al., 1993). These receptors have been cloned and are encoded by separate genes (Arai et al., 1990; Sakurai et al., 1990). The ET_B receptors have been tentatively classified into two groups, ET_{B1} and ET_{B2} depending on their response to receptor antagonists; how these relate to the cloned ET_B receptor is not yet known (Douglas et al., 1994; Pollock et al., 1995). Both subtypes have been found in

brain (Sokolovsky et al., 1992). A third receptor that preferentially binds ET-3 has been reported in Xenopus dermal melanophores and named ET_C but it is unclear whether this is a species homologue of ET_A or ET_B receptors (Karne et al., 1993). The receptors are all members of the G-protein-coupled seven transmembrane domain superfamily and can activate a number of signal transduction pathways (Nambi et al., 1995). The effects of endothelin binding to the receptors include activation of phospholipases C, D and A_2, generation of inositol-1,4,5-trisphosphate and DAG, influx of Ca^{2+} through opening of Ca^{2+} channels, activation and inhibtion of adenylate cyclase by ET_A and ET_B respectively, guanylate cyclase activation, and protein kinase C activation (Nambi et al., 1995) The pathway activated depends on the cell type and the receptor subtype.

The receptor subtypes present can be distinguished by use of peptide antagonists or agonists that have receptor specificity. Two antagonists have been used in the present study: BQ-123 and IRL 1038. BQ-123 is a cyclic pentapeptide that is highly specific for ET_A receptors and acts as a competitive antagonist (Ihara et al., 1992; Vigne et al., 1993). IRL 1038 is an ET_B receptor-selective antatgonist and is an 11 amino acid peptide corresponding to the C-terminal portion common to all ETs, with a single disulphide link between Cys^{11}-Cys^{15}([Cys^{11}-Cys15]-ET-1(11-21)) (Urade et al., 1992).

ET-1, but not ET-2 or -3 is produced by endothelial cells (Ohlstein and Ruffolo, 1995) while ET-3 has been reported to be the major endothelin produced by neuronal tissue (Willette et al., 1995). Many cell types synthesize endothelins, including vascular smooth muscle, bronchial smooth muscle, mesangial cells, and fibroblasts (Ohlstein and Ruffolo, 1995). The initial studies of endothelin action were on the cardiovascular system where action of ET-1 via ET_A receptors lead to potent vasoconstriction by stimulating smooth muscle contraction. Both ET-1 and ET-3 were equipotent for ET_B receptor activation and lead to vasodilation. However, it is now clear that ET_B receptors are also responsible for smooth muscle contraction including vascular smooth muscle (Bax and Saxena, 1994). ETs have also been implicated in renal function, pulmonary bronchoconstriction and tone of non-vascular smooth muscle; they also appear to function as endocrine hormones affecting the adrenal glands, thyroid and parathyroid glands and may play a role in smooth muscle contraction during pregnancy (Ohlstein and Ruffolo, 1995).

Endothelins have growth factor activity, acting as potent mitogens for many cell types including vascular smooth muscle cells (Komuro et al., 1988), 3T3 fibroblasts (Takuwa et al., 1989), mesangial cells (Simonson and Herman, 1993), melanocytes (Imokawa et al., 1992) and astrocytes (MacCumber et al., 1990). ET-1 stimulates c-fos and c-myc expression in vascular smooth muscle cells (Komura et al., 1988) and fibroblasts (Takuwa et al., 1989) and c-fos and c-jun expression in C6 glioma cells (Yin et al., 1992). As well as being mitogens for astrocytes, endothelins regulate gap junctions between the cells, causing gap junction closure (Giaume et al., 1992).

In the CNS, endothelins are produced by neurones and endothelial cells *in vivo* (Willette et al., 1995) and can be produced by astrocytes *in vitro* (MacCumber et al., 1990). ETs are widely distributed throughout the adult human brain, the major areas of ET production being the hypothalamus, the pituitary and the spinal cord (Takahashi et al., 1991). Northern blot analysis of adult rat brain has revealed that ET-3 is the predominant isoform (MacCumber et al., 1990). ETs have been localized by immunocytochemistry to neurones in the dorsal horn of the adult human spinal cord (Giaid et al., 1989) and produce depolarization of newborn rat spinal motorneurones (Yoshizawa et al., 1989) indicating that they can function as neuromodulators. In the PNS, ET immunoreactivity has been detected in adult human DRG neurones (Giaid et al., 1989) and in neonatal rat myenteric neurones (Eaker et al., 1995).

Both main subtypes of receptor are expressed on neurones in the brain, the distribution of subtype depending on the type of neurone, while astrocytes appear to have predominantly the ET_B receptor (Willette et al., 1995). Binding sites for ET-1 have been shown throughout the developing spinal cord, localizing to the grey matter in adult rat (Kar et al., 1991). ET-1 also localises to glial cells of the rat DRG, with no significant change in binding during postnatal development (Kar et al., 1991) but the exact receptor subtype present on these cells has not been determined.

Mouse mutants have been made that are deficient in ET-1, ET-3 or the ET_B receptor. Mice deficient in ET-1 have elevated blood pressure, and homozygotes have extensive craniofacial abnormalities, the latter effect is possibly a result of deficiencies in cranial neural crest migration or survival (Kurihara et al., 1994). Mice deficient in ET-3, have a similar phenotype to mice that have mutant ET_B receptors (Greenstein Baynash et al., 1994; Hosoda et al., 1994). These mice lack myenteric ganglia in the colon and

have white-spotted coat colour as a result of an absence of melanocytes, seemingly due to loss of neural crest in the absence of ET signalling. Thus ETs play a role in development of some derivatives of the neural crest *in vivo*.

This Chapter presents data on the survival effects of NDFβ-2 and ETs on Schwann cell precursors. Both NDFβ-2 and ET-1 can act as long term survival factors but only NDFβ-2 makes the precursors mature into cells with a Schwann cell phenotype. In the presence of ET-1, the precursors do not mature and ET-1 actively blocks the maturation effects of NDFβ-2 *in vitro*.

RESULTS

In Chapters 3-5, p75LNGFr was used as a marker for Schwann cell precursors. As described in Chapter 3, Z. Dong has shown that 3-5% of cells that express this antigen do not express the L1 antigen and are morphologically distinct (Z. Dong, personal communication). In this Chapter, L1 was used as the marker for cells of the Schwann cell lineage in the survival assays; p75LNGFr expression was used when double-labelling for S100 expression, since both S100 and L1 used biotin-conjugated second layers and were therefore incompatible (see Chapter 2).

NDFβ-2 promotes Schwann cell precursor survival

NDFβ-2 was used in the 20 hr survival assay in the presence of low levels of insulin (5.7 ng/ml, 1nM) and 100ng/ml IGF-1 (13nM); at the end of this time surviving precursors were determined by staining with the L1 antibody (Fig 6.1). NDFβ-2 promoted the survival of the precursors in a dose dependent manner, with an EC_{50} of 0.25ng/ml, ~ 10pM (Fig 6.2). Survival of 100% was obtained with concentrations of 1ng/ml and above, although at higher concentrations the survival was greater than 100%, indicating that NDFβ-2 was mitogenic for the cells.

NDFβ-2 is a long term survival factor for precursors

To determine whether NDFβ-2 could promote long term survival of these cells, precursors were cultured on coverslips in 1.5ng/ml (60pM) NDFβ-2 and stained for L1 at 20, 44, 68, and 92 hr after plating. The medium was replaced every day and contained freshly thawed growth factors. The survival of the cells after the extended period in culture was compared to the number of cells that had attached, flattened and were L1 positive at 3 hr post-plating. At 20 hr, 98% of cells survived (Fig 6.3); the cell numbers then proceeded to increase with time, and by 92 hr after plating, the starting population had increased by ~20%. This increase in number is a further indication that not only is NDFβ-2 a long term survival factor but is also a mitogen for these cells.

The survival effects of NDFβ-2 seemed to be specific for Schwann cell precursors. At 20 hr, between 12 and 18% of cells in the cultures did not express L1 and therefore were not precursors. It was observed that the number of contaminating cells did not

increase with increased time in culture; after 4 days the number of L1 negative cells were less than 10% of the total starting population.

NDFβ-2 promotes the maturation of Schwann cell precursors *in vitro*

It had been shown previously that Schwann cell precursors cultured in the presence of bFGF and 1% serum or NCM would survive long term and mature into cells that could survive culture in defined medium and express the S100 protein (Jessen et al., 1994). Since NDFβ-2 could promote long term survival of the precursors, the effect of this growth factor on the maturation of the precursors was studied. Three parameters were used to determine the extent of the precursor maturation: S100 expression, survival of the cells in defined medium after plating at moderate density, and their response to bFGF and forskolin; as described in Chapter 5, this latter combination is mitogenic for Schwann cells but does not promote DNA synthesis in precursors.

<u>S100 expression and survival</u>

Nerves from E14, E15, E17, E18 and newborn rats, were dissociated and plated, as described in Chapter 2, in laminin coated tissue culture wells with defined medium containing 1nM insulin and 100ng/ml IGF-1, together with 1.5 ng/ml (60pM) NDFβ-2. The medium was replaced every day with fresh medium containing newly thawed growth factors. The cells were cultured from 1-4 days before replating in defined medium containing insulin and IGF-1 only, thus removing any influence from growth factors that may have become associated with the original substrate. In order to compare the S100 expression and survival in defined medium to that seen in cells plated directly from the nerves, the data given in Chapter 3, Figs 3.3 and 3.6, has been reproduced here.

When cells were replated after 1 day *in vitro*, those from E14 + 1 (=E15) cultures showed very low levels of survival, comparable to the survival seen in cells from E15 nerves (Table 6.1). Cells from E15 + 1 (=E16) cultures showed somewhat higher survival, in line with the increased survival seen in cells directly from E16 nerves. By E18, cells taken directly from the nerve showed more than 90% survival in the assay and this was also seen in cells from E17 + 1 (=E18). Newborn nerves when cultured for 1 day and then replated showed 80% survival, somewhat lower than that seen in cells taken directly from the nerves.

When E14 cells were cultured for 2 days before replating (=E16) survival was similar that seen in cells from E16 and E15 + 1, and was higher than that seen after 1 day *in vitro*. After 3 days *in vitro* (=E17), survival had increased to around 71%, although this was not quite as high as that seen in cells from E17 nerves. A further day *in vitro*, E14 + 4 (E=18) increased the survival after replating to ~85%, similar to the number surviving in cultures from newborn + 1 day, and only slightly less than that observed in cells from E18 nerves and E17 +1 cells.

The expression of S100 immunoreactivity in the cytoplasm of the cells increased with time in culture in a similar manner: few cells were positive for S100 at E14 + 1 (=E15) but this had increased to almost half the cells at E14 + 2 (=E16) and E15 + 1 (=E16), paralleling the expression seen in the developing nerve (Table 6.1). After 3 days *in vitro*, E14 + 3 (=E17) the number of cells expressing S100 had increased to 75%, slightly lower than that seen in the nerve at E17 (95%). The expression of S100 at E14 + 4 (=E18) had increased further to 84%, but was again slightly lower than that seen in cells taken from the nerve at E18 (96%). An example of the type of S100 staining is shown in Figure 6.4 C, with cells exhibiting varying levels of S100 expression (note cell indicated by double arrow). The S100 expression started as a nuclear stain and rapidly spread to the surrounding cytoplasm. A cell not expressing p75LNGFr or S100, and likely to be a contaminating cell not of the Schwann cell lineage, is indicated by the asterisk.

To determine whether the cells surviving at 20 hr after plating all expressed S100, cells that had been cultured in NDFβ-2 for 4 days were replated and cultured in defined medium with 1nM insulin and 100ng/ml IGF-1 for 20 hr after replating before staining for p75LNGFr and S100 expression. Of the surviving cells, 90% ± 1 (SD, n=2) expressed both p75LNGFr and S100; however, the remaining 10% of cells expressed p75LNGFr but were S100 negative.

Response to bFGF and forskolin

The mitogenic effects of bFGF and forskolin on cells taken directly from embryonic and neonatal nerves had previously been determined by Z. Dong (Dong et al., 1995). This work revealed that cells from E14 and E15 nerves initially showed levels of division that reflected proliferation found *in vivo* in the developing nerve (Stewart et

al., 1993), but that these levels fell dramatically after 5 hr culture in the presence of bFGF and forskolin (Fig 6.5 A). However, by E17 this combination of growth factors could maintain initial levels of DNA synthesis, and induced higher levels of DNA synthesis in cells from E18 and newborn rats.

To determine whether the precursors became responsive to the mitogenic effects of these factors after culture in NDFβ-2, cells were replated in defined medium with 1nM insulin and 100ng/ml IGF-1 and were dosed with 10ng/ml bFGF and 5μM forskolin (in the presence of 100ng/ml IGF-1 and 1nM insulin) after 3 hr in culture. The cells were then pulsed with BrdU for 1.5 hr after 3.5, 13.5 and 18.5 hr in culture and stained for BrdU incorporation and L1 expression at 5 hr, 15 hr and 20 hr in culture, repeating the method used for the measure of DNA synthesis in cells directly from the nerve (Dong et al., 1995). Cells from E14 nerves replated after 1 day (=E15) showed a decrease in DNA synthesis over the 20 hr experimental period (Fig 6.5 B). However, cells from E14 + 3 (=E17) and E14 + 4 (=E18) both showed an increase in DNA synthesis in the presence of bFGF and forskolin, indicating that these cells had acquired the ability to respond to these factors.

Morphology

The transition of precursors to Schwann cells involves a change in the morphology observed *in vitro*, from flattened groups of cells with extensive cell contacts at E14 and E15 to bi- and tripolar cells observed in Schwann cells taken from E17 nerves and older (Fig 3.2). Cells from E14 nerves cultured in NDFβ-2 initially formed groups similar to those seen at 3 hr post-plating (Fig 6.1). However, with extended time in culture in NDFβ-2, the cells became greatly flattened with the limits of each cell being increasingly difficult to determine by phase microscopy (Fig 6.10 A). When E14 cells were cultured for 4 days in 1.5ng/ml NDFβ-2, 1nM insulin and 100ng/ml IGF-1 and then replated into medium containing 1nM insulin and 100ng/ml IGF-1, the cells initially adopted a flattened morphology with only a few cells at 3 hr extending processes. However, by 20 hr after replating, many cells had extended processes (Fig 6.6). Many cells adopted bi-, tri- and multipolar morphologies, but the cytoplasm in the region of the nucleus continued to be flattened, unlike Schwann cells taken directly from E18 nerves (Fig 3.2). A few cells in the replated cultures failed to extend any processes, remaining flattened as they had been prior to replating.

Maturation of Schwann cell precursors does not require DNA synthesis

DNA synthesis was examined in precursors cultured in 1.5ng/ml NDFβ-2 using a 1.5 hr pulse of BrdU on each of days 1-4 and was found to be between 10-20%, comparable to that seen *in vivo* during the developmental period E14-E18 (Stewart et al., 1993). To determine whether DNA synthesis is absolutely required for the precursors to acquire the Schwann cell phenotype, cells were cultured in a concentration of NDFβ-2 (0.7 ng/ml, 28pM) that promoted survival but induced only low levels of division in the precursors. When division was measured in sister cultures in the 3 days prior to replating, using a 1.5 hr pulse of BrdU at the end of each culture period, this concentration of NDFβ-2 produced DNA synthesis of 1% ± 0.4 (SD, n=2), 2% ± 1 (SD, n=2), and 2% ± 1 (SD, n=2) on days 1, 2 and 3 *in vitro* respectively. After replating on day 4, cytoplasmic S100 was detected in 89% ± 5 (SD, n=2) of the cells at 3 hr post-replating and 67% ± 1 (SD, n=2) of L1 positive cells survived at 20 hr in defined medium. These cells also responded to bFGF and forskolin, with DNA synthesis increasing from 3% ± 0 (SD, n=2) at 5 hr to 13% ± 0.4 (SD, n=2) at 20 hr. Thus, in conditions where DNA synthesis was low, the precursors could still generate Schwann cells.

To confirm that Schwann cells could be generated without cell division, precursors were cultured in a lower concentration of NDFβ-2 (0.4ng/ml, 16pM), in the continuous presence of BrdU for 3 days. At the end of this time they were replated and double labelled for BrdU and L1 at 3 hr and assayed for survival in defined medium at 20 hr. Only 41% ± 2 (SEM, n=3) of the cells that survived at 20 hr were double labelled, indicating that more than half of the surviving cells had not undergone division during the 4 day culture period. The number of cells surviving, however, were similar to those seen in assays with higher concentrations of NDFβ-2 (see Table 6.1) with 78% ± 14 (SEM, n=3) surviving in defined medium at 20 hr. At 3 hr the number of cells expressing S100 was 63% ± 10 (SEM, n=3).

Endothelins block Schwann cell precursor death

When used in the 20 hr survival assay endothelins (ET) 1, 2 and 3 promoted precursor survival in defined medium in the presence of 100ng/ml IGF-1 (Fig 6.7 A, B, C). All three peptides acted in a similar dose-dependent mannner, maximally promoting 80% survival at the most optimal concentrations. The EC_{50} for ET-1 and ET-3 were

identical at ~0.02nM , with ET-2 slightly higher at 0.05nM. The survival required the presence of IGF-1, no survival was observed in the absence of this growth factor (not shown).

The morphology of E14 cells cultured for 20 hr with ETs in the presence of 1nM insulin and 100ng/ml IGF-1 (eg. ET-1; Fig 6.8) was similar to that seen in cultures of NDFβ-2 (Fig 6.1). Flattened pavements of cells were observed with extensive cell contacts.

The similar effects of all three ETs suggested that their actions were via the ET_B receptor. Antagonists for both the ET_A and ET_B receptor were used to determine which type of receptor was present on the precursors. The antagonist for ET_A receptors BQ-123 produced no decrease in survival when used in the concentration range 0.1nM - 10μM in the presence of 0.3nM ET-1. Surprisingly, when the ET_B antagonist IRL 1038 was used in the concentration range 10pM - 1μM also in the presence of 0.3nM ET-1, there was no decrease in survival at any of the antagonist concentrations.

Endothelin-1 promotes long term survival of precursors

To determine whether ETs could promote long term survival of precursors, cells were cultured in 0.3nM ET-1 in the presence of 1nM insulin and 100ng/ml IGF-1 for 1-4 days, as described for NDFβ-2. Medium containing fresh growth factors was replaced daily. The cells were stained for L1 at 20, 44, 68 and 92 hr after plating and the surviving cell number compared to the number of attached and flattened L1-positive cells at 3 hr post-plating. After 20 hr in culture, 72% of the cells were alive (Fig 6.9). The number of cells surviving decreased to 40% of the starting population over the 92 hr culture period, suggesting that although ET-1 is a long term survival factor for some of these cells it is unable to promote survival of all cells and is not mitogenic (Fig 6.9). Pulsing the cells with BrdU for the last 1.5 hr in culture over 4 days showed a low level of DNA synthesis, between 1-3%, at each time point.

ET-1, like NDFβ-2, did not appear to support long term survival of contaminating L1-negative cells. At the start of the cultures, a higher proportion of contaminating cells were found in cultures with ET-1; 20-23% of the cells after 20 hr in culture were L1-negative, compared with 12-18% of L1-negative cells in NDFβ-2 at the same

timepoint. However, this number decreased with time, so that by 4 days, similar numbers of these cells were present in ET-1 cultures compared to NDFβ-2 cultures (less than 10% of the total starting population).

Endothelin-1 does not promote precursor maturation

Since NDFβ-2 promoted the maturation of the precursors in culture the question arose whether the precursor / Schwann cell transition was a consequence of long term survival or whether the maturation of the cells was a response to a particular growth factor.

Cells from E14 nerves were cultured for 4 days in 0.3nM ET-1 with 1nM insulin and 100ng/ml IGF-1 and then replated as previously described. S100 expression was determined at 3 hr and survival in defined medium at 20 hr. It was observed that of the replated cells very few had matured to the Schwann cell phenotype. At 3 hr, only 16% ± 2 (SD, n=2) of the replated cells expressed cytoplasmic S100 and the survival at 20 hr was 17% ± 1 (SD, n=2). In parallel experiments in the presence of 1.5ng/ml NDFβ-2, 1nM insulin and 100ng/ml IGF-1, 87% ± 1 (SD, n=2) of the replated cells expressed S100 and 92% ± 4 (SD, n=2) were alive at 20 hr in defined medium (Fig 6.11).

Unlike cells cultured in NDFβ-2, the morphology of cells cultured with ET-1 did not change appreciably with time in culture (compare Fig 6.8 with Fig 6.10 C, D). After 4 days in culture the cells were still in close groups, however the extensive flattening observed in NDFβ-2 cultures did not occur in ET-1 (compare Fig 6.10 A, B with Fig 6.10 C, D). In some cultures, particularly when high concentrations (>10nM) of ET-1 were used, the cells appeared to contract together after 3-4 days in culture, forming dense clumps of cells (not shown).

Endothelin-1 can block the maturation effect of NDFβ-2 on precursors

The lack of maturation of the precursors in ET-1 was unexpected. Suppression of cell death by ET-1 appeared to be insufficient to allow maturation of these cells in culture. To determine whether the lack of maturation observed in cultures with ET-1 was due to an inability of ET-1 to promote precursor maturation, or whether ET-1 actively prevented the transition of precursors to Schwann cells, cultures were prepared that contained both 0.3nM ET-1 and 1.5ng.ml (60nM) NDFβ-2. After 4 days *in vitro* with

medium changed every day, the cells were replated. The expression of S100 at 3 hr was 38% ± 10 (SD, n=2) and survival in defined medium was 50% ± 8 (SD, n=2).

Table 6.1 Maturation of Schwann cell precursors cultured in NDFβ-2

Cells from different age nerves were cultured for various times *in vitro*: + 0, indicates experiments where the survival assays and S100 expression were measured on cells taken directly from the nerves; the data here has been presented earlier as Figs 3.3 and 3.6. These cells were cultured in defined medium containing 1μM insulin.

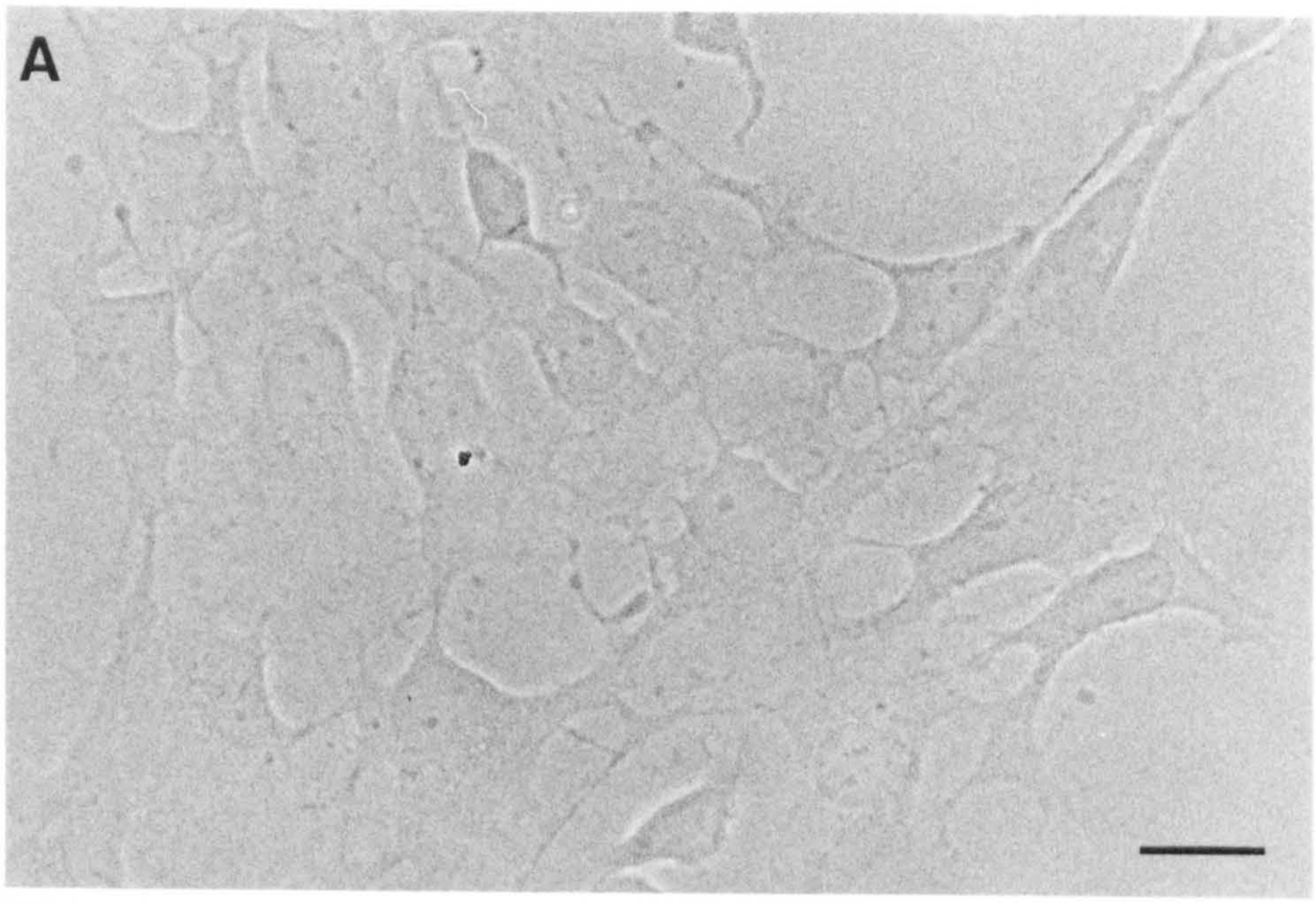
+ 1-4 days *in vitro* indicates the time cells were cultured in defined medium with 100ng/ml IGF-1, 1nM insulin and 1.5ng/ml (60pM) NDFβ-2 prior to replating in this medium but without NDFβ-2. The figures represent data from three experiments except for that of newborn cells which was from two experiments; the error for these latter experiments is the standard deviation.

Table 6.1 Maturation of Schwann cell precursors cultured in NDFβ-2

Cells - Age when removed from nerve + time *in vitro*	Percentage of cells that express S100 ± SEM	Survival percentage ± SEM
E14 + 0	4 ± 3	1 ± 2
E15 + 0	4 ± 4	3 ± 2
E14 + 1	15 ± 2	12 ± 2
E16 + 0	42 ± 6	16 ± 5
E14 + 2	41 ± 4	30 ± 3
E15 + 1	47 ± 7	18 ± 3
E17 + 0	95 ± 2	87 ± 5
E14 + 3	75 ± 3	71 ± 2
E18 + 0	96 ± 2	98 ± 2
E14 + 4	84 ± 3	85 ± 9
E17 + 1	93 ± 1	92 ± 6
Newborn + 0	93 ± 6	100 ± 4
Newborn +1		79 ± 5

Figure 6.1 Precursor morphology and L1 expression after 20 hr culture in NDFβ-2.

E14 precursors were cultured in defined medium containing 1.5ng/ml (60pM) NDFβ-2, 1nM insulin and 100ng/ml IGF-1 for 20 hr and then stained for L1 expression. (A) Phase contrast micrograph of the cells represented in (B). (B) L1 expression on cells cultured in NDFβ-2 for 20 hr. The morphology of the cells under these culture conditions closely resembled that of cells cultured for 20 hr with NCM (Fig 3.5 C) or bFGF (Fig 5.2). All precursors expressed L1. Bar = 20μm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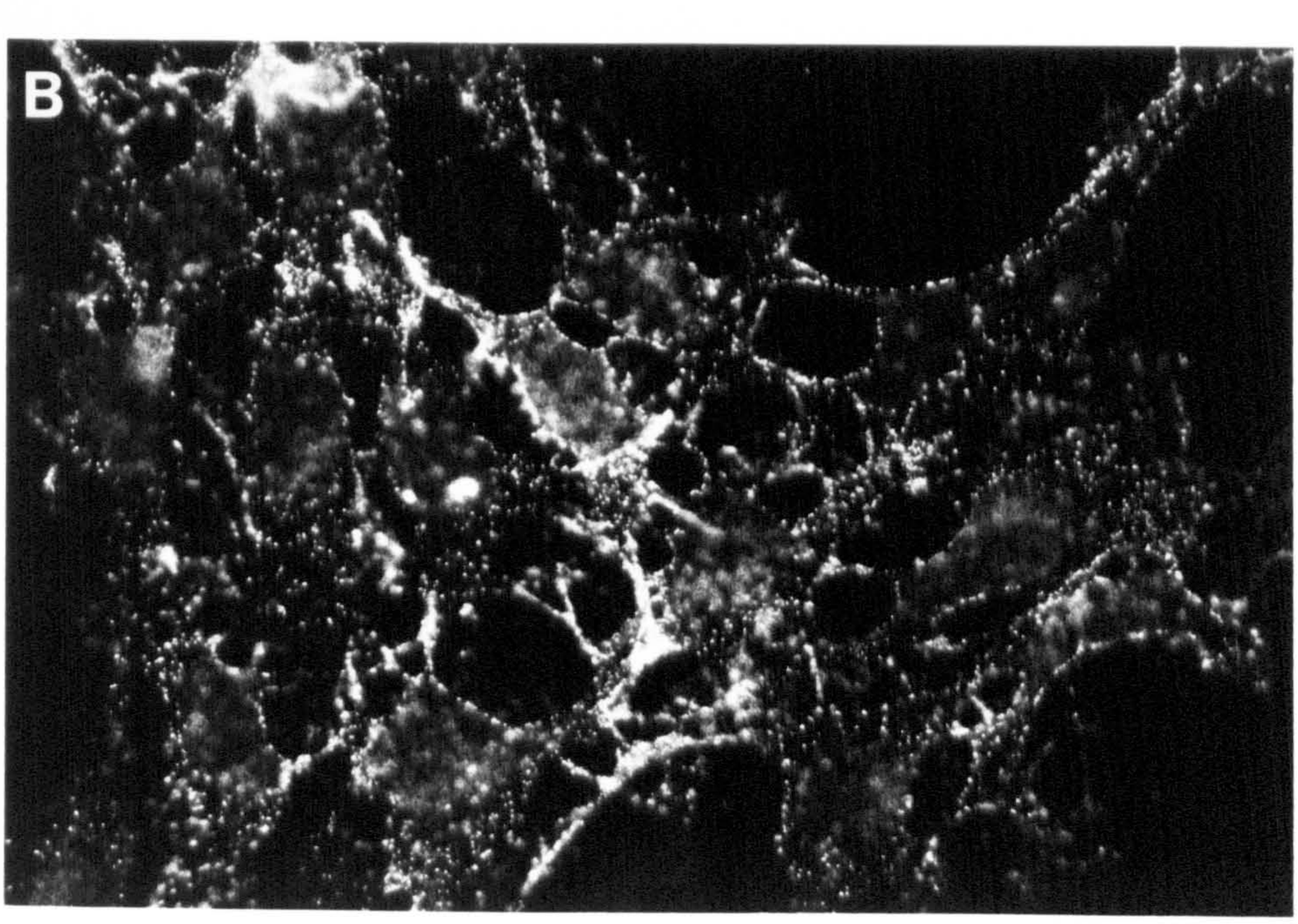

B

Figure 6.2 NDFβ-2 promotes precursor survival

E14 precursors were cultured in the presence of increasing concentrations of NDFβ-2, 1nM insulin and 100ng/ml IGF-1. At 20 hr the cells were fixed and stained for L1, and the percentage survival determined as described previously. Survival of precursors increased in a dose-dependent manner. Each point represents the average from three experiments, error bars indicate SEM.

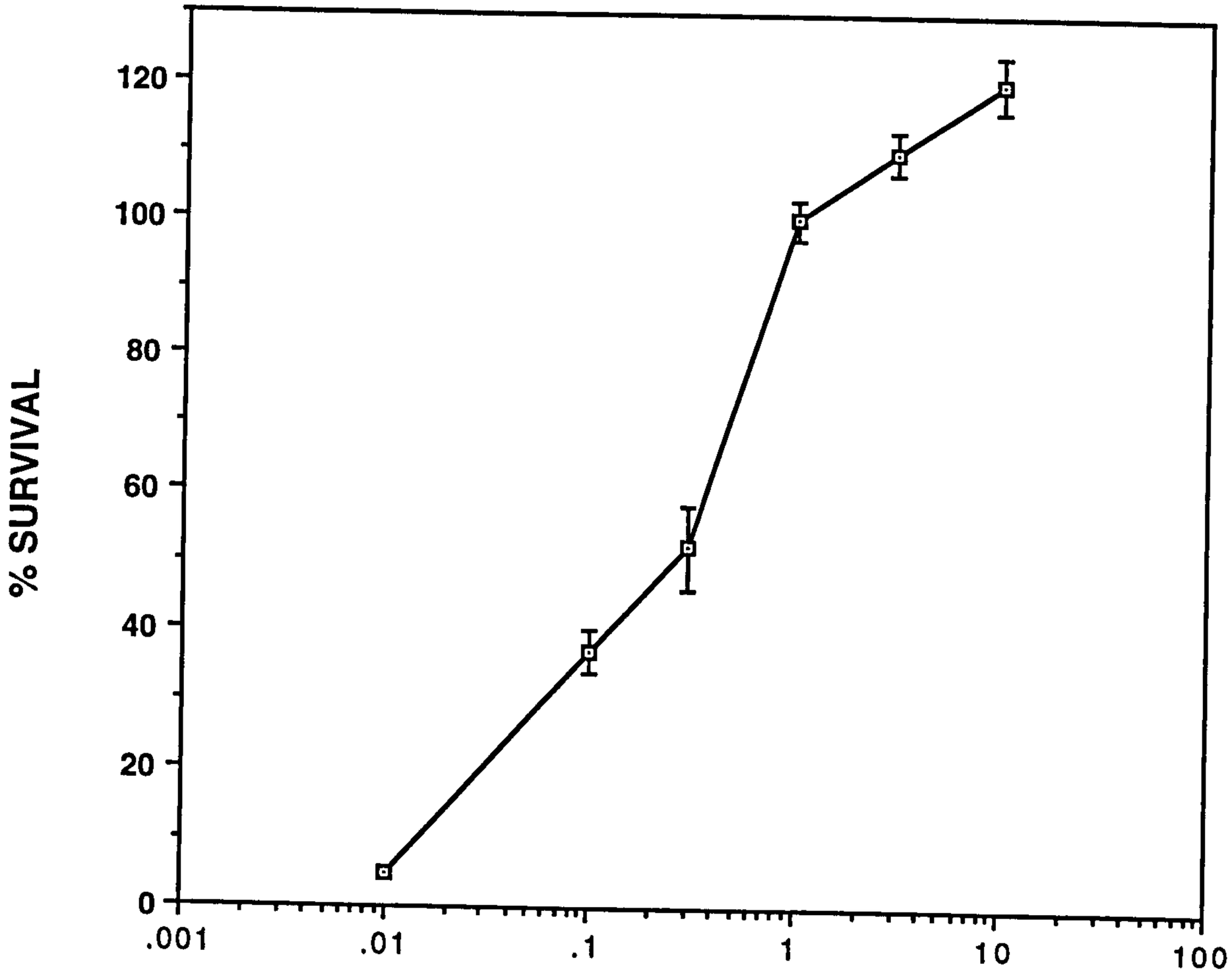

% SURVIVAL
120
100
80
60
40
20
0
.001
.01
.1
1
10
100
CONCENTRATION (ng/ml)

Figure 6.3 NDFβ-2 promotes long term survival of precursors

E14 cells cultured in 1.5ng/ml (60pM) NDFβ-2, 1nM insulin and 100ng/ml IGF-1 were stained for L1 at 20, 44, 68 and 92 hr after plating. The percentage survival was calculated relative to L1 positive cells that had attached and flattened 3 hr post-plating. NDFβ-2 is able to prevent precursor death in the long term and is mitogenic for these cells. Each point represents the average of two experiments, error bars indicate SD.

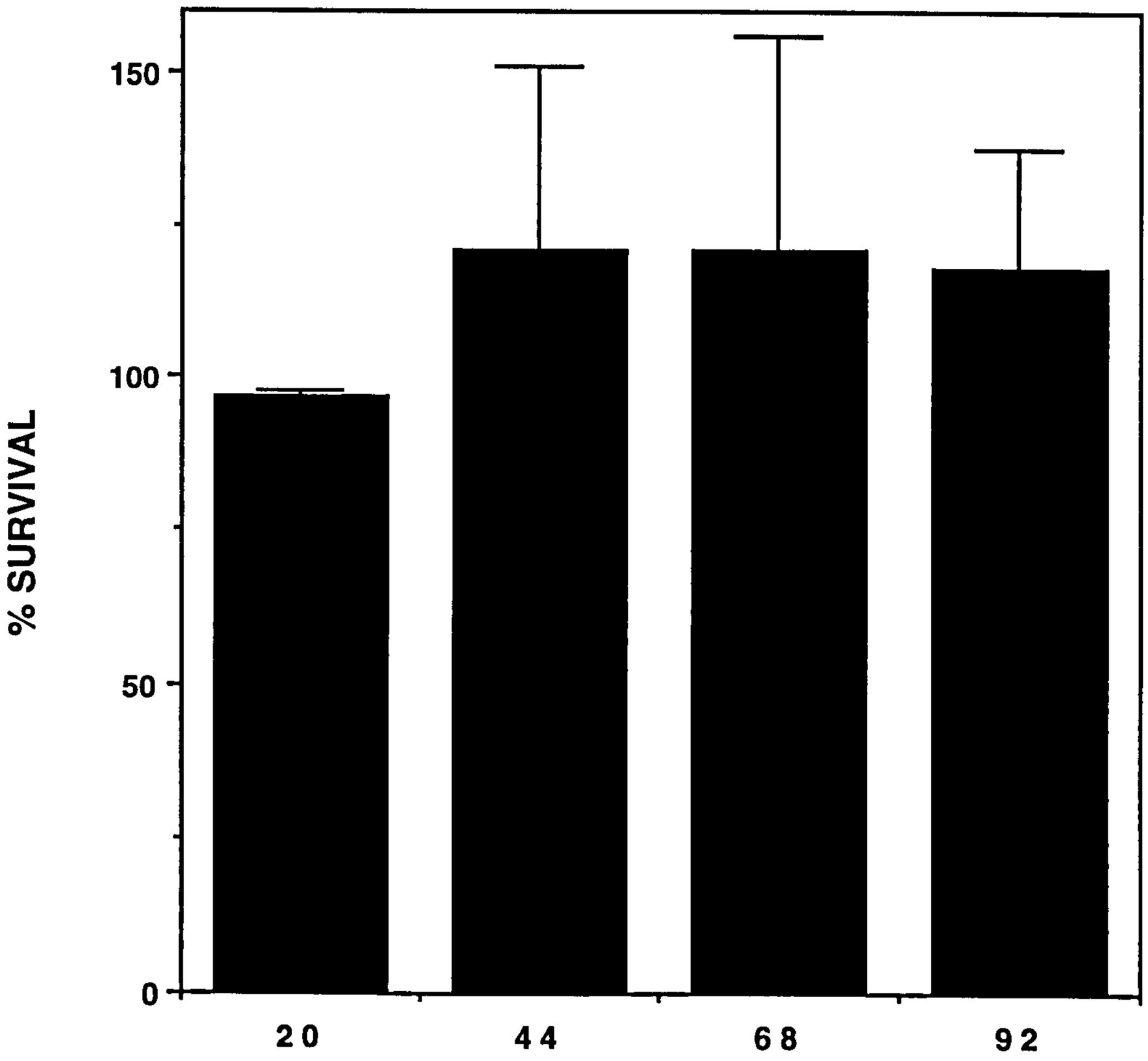
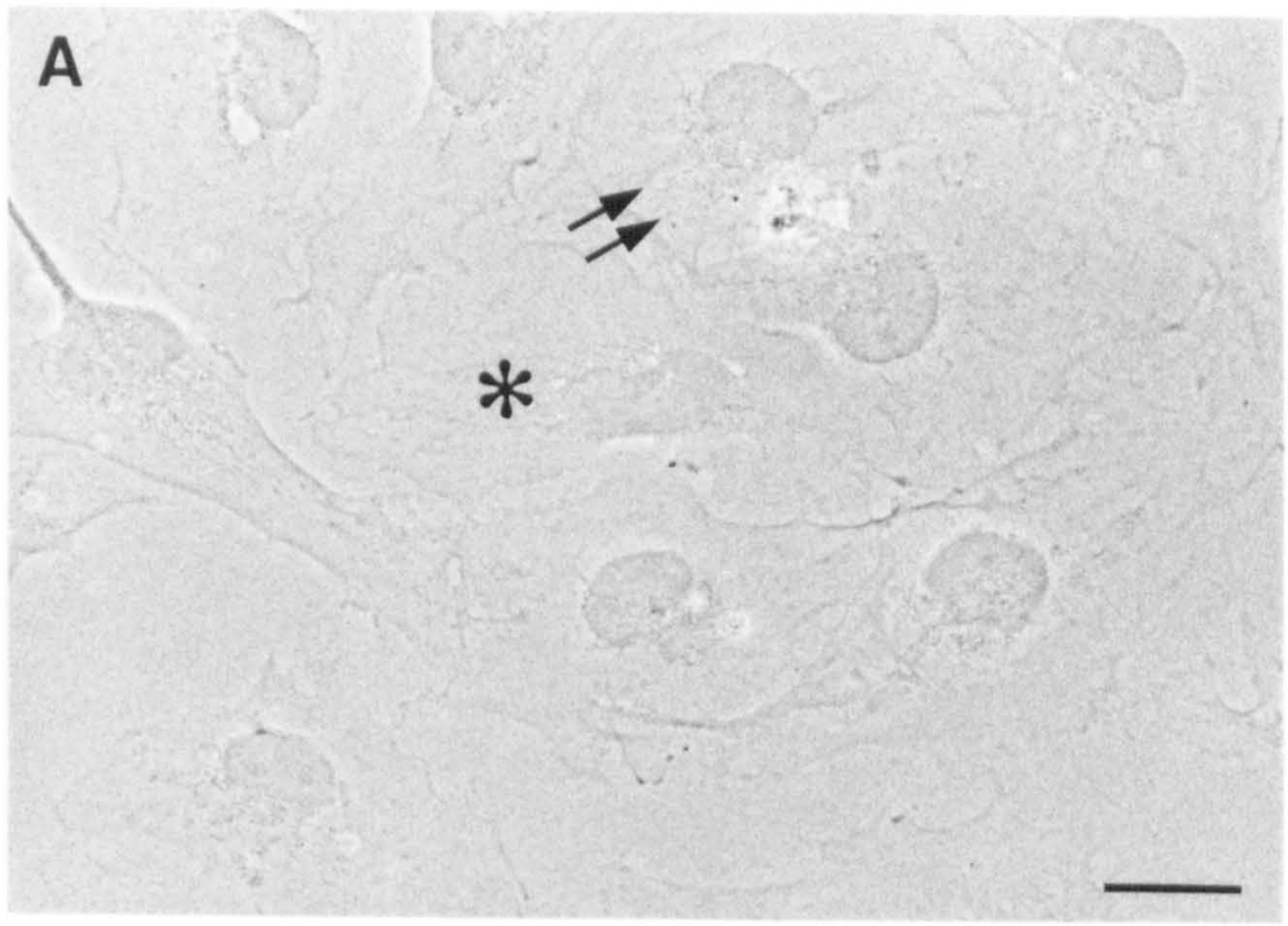

150
100
50
0
% SURVIVAL
20
44
68
92
TIME AFTER PLATING (hours)

Figure 6.4 Cells from E14 rats express S100 after 4 days in culture with NDFβ-2

E14 cells cultured on LN coated tissue culture wells in 1.5ng/ml NDFβ-2, 1nM insulin and 100ng/ml IGF-1 for 4 days were replated onto LN coated glass coverslips and stained at 3 hr post-replating. (A) Phase contrast view of cultures represented in (B) and (C); (B) p75LNGFr staining with 192Ig antibody; (C) S100 expression. Most cells exhibit S100 labelling, the level of this varies between cells; double arrows indicate a cell expressing low levels of S100. Asterisk indicates a non-precursor-derived cell that does not express either p75LNGFr or S100. Bar = 20μm.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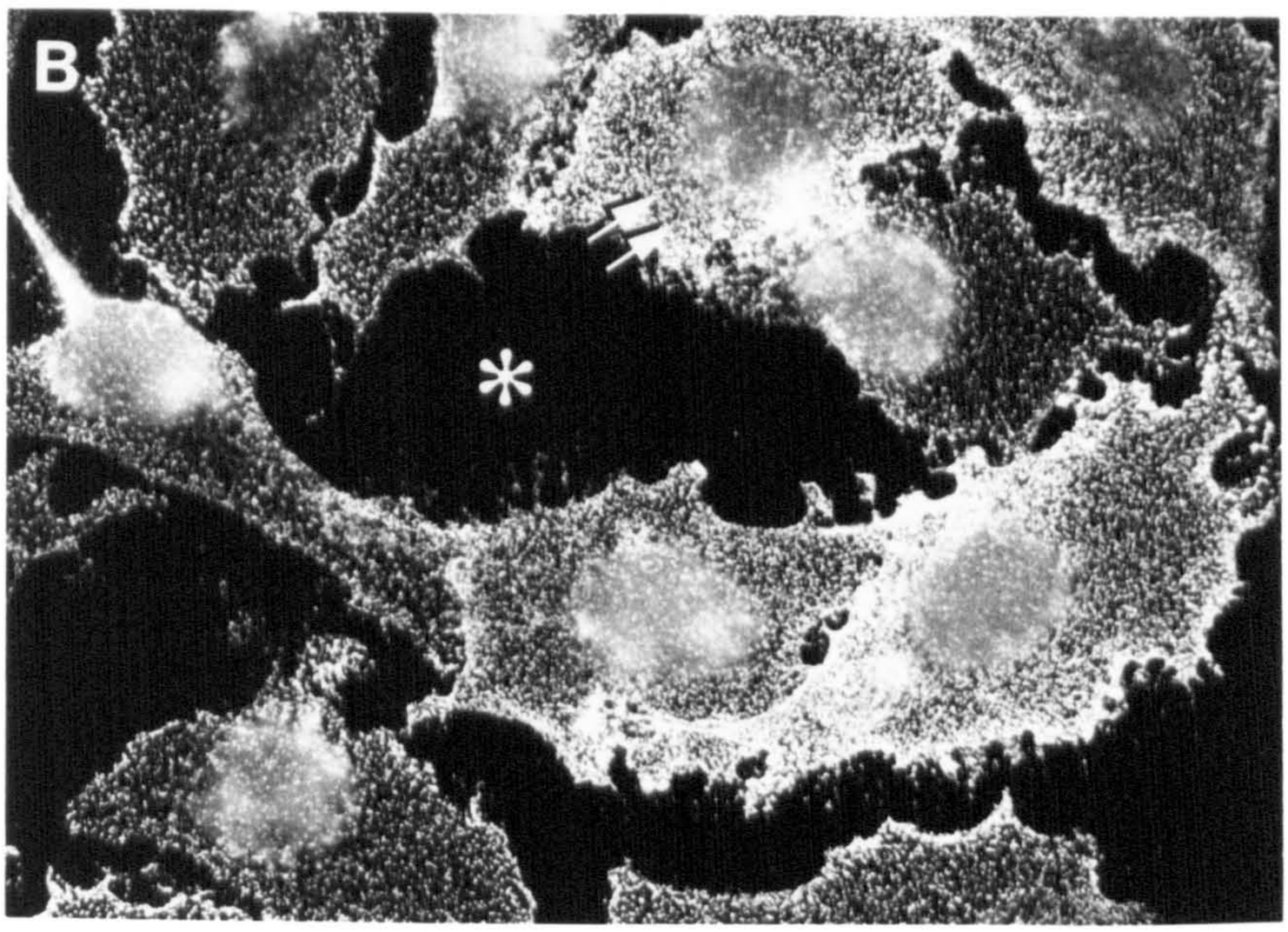
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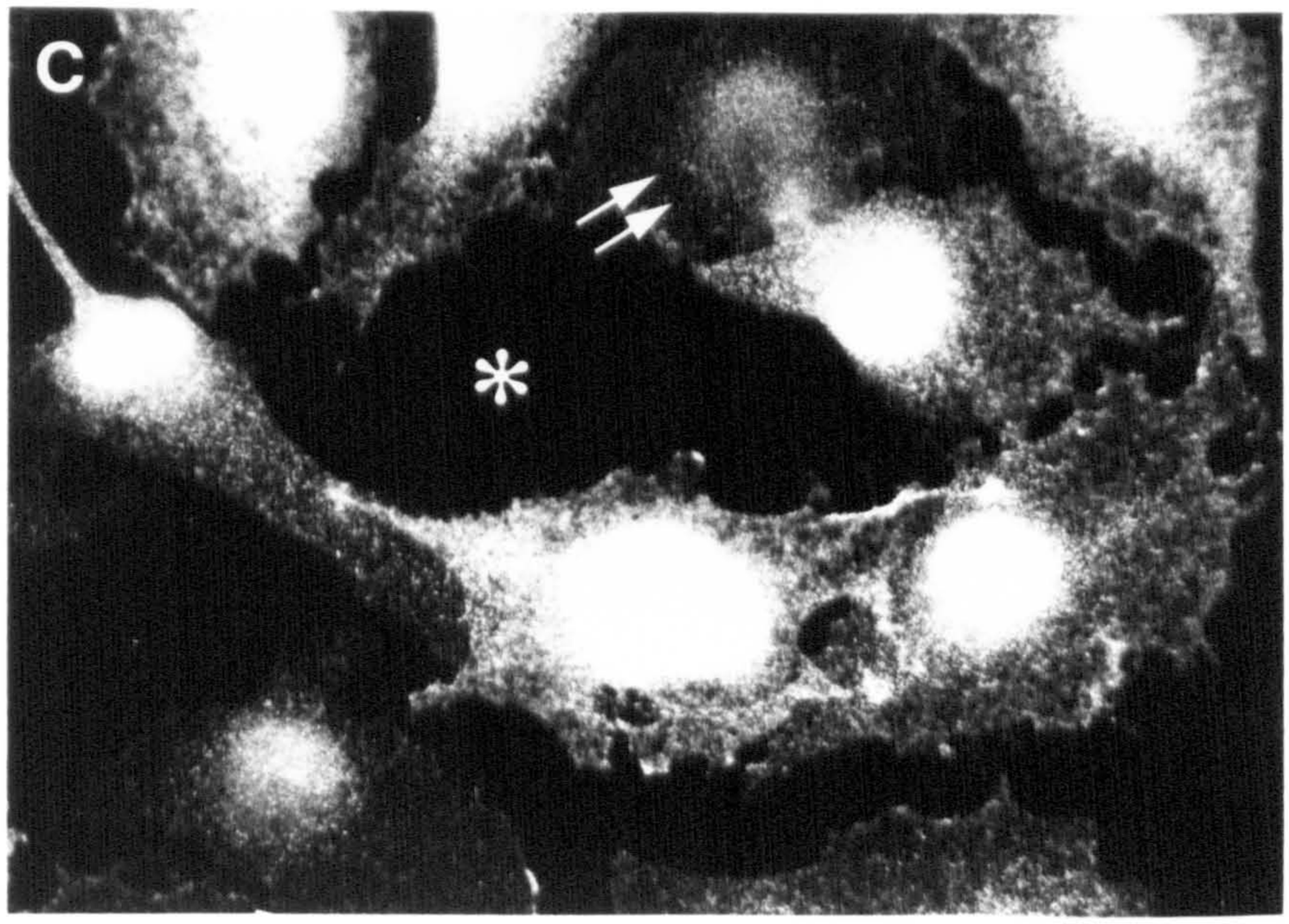
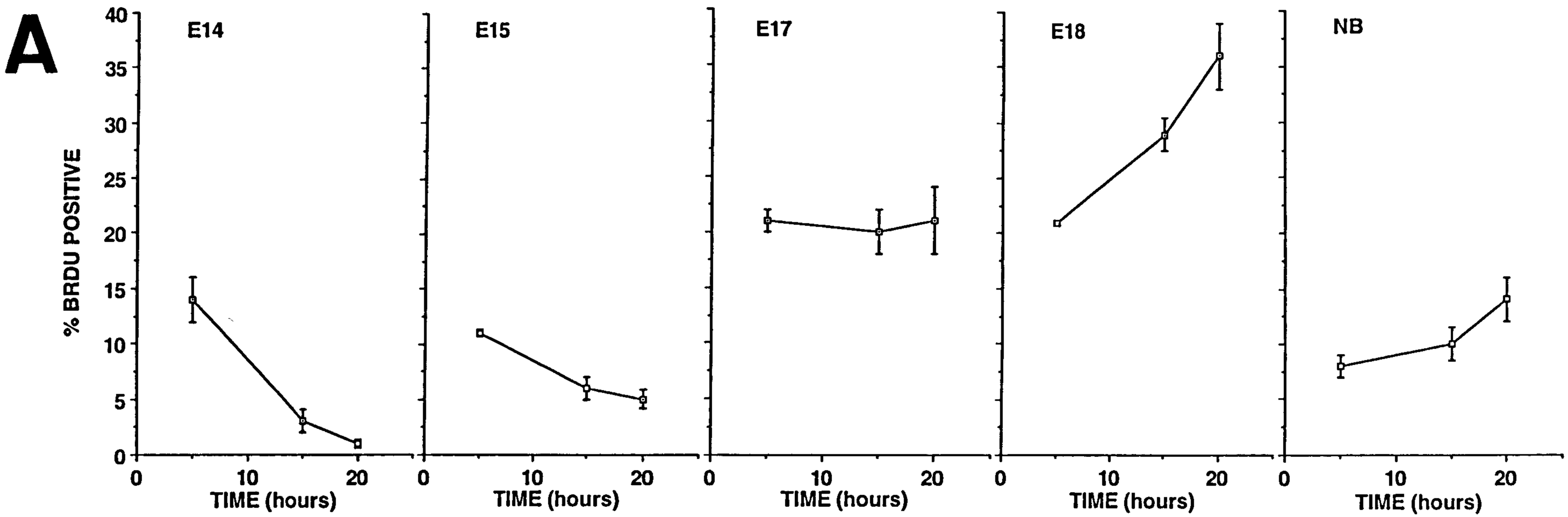

C
*

Figure 6.5 A mitogenic response to bFGF plus forskolin distinguishes Schwann cells from precursors

(A) The graphs show the percentage of L1-positive cells that incorporated BrdU when taken directly from the nerve and cultured in 10ng/ml bFGF and 5μM forskolin, with BrdU pulsing for 1.5 hr at 5, 15 and 20 hr after plating. A response to these Schwann cell mitogens is first apparent at E17, when cells with a Schwann cell phenotype are first seen in the nerve (Chapter 3). These data are from work by Z. Dong.

(B) The same assay was performed on precursors cultured for various times in NDFβ-2 and replated. The mitogenic effect of bFGF and forskolin is first apparent at E14 + 3, a time equivalent to E17 *in vivo* and similar to that seen in cells taken directly from the nerve at that age. All points are an average of 3 experiments, error bars indicate SEM.


A
E14
E15
E17
E18
NB
% BRDU POSITIVE
0
5
10
15
20
25
30
35
40
0
10
20
TIME (hours)

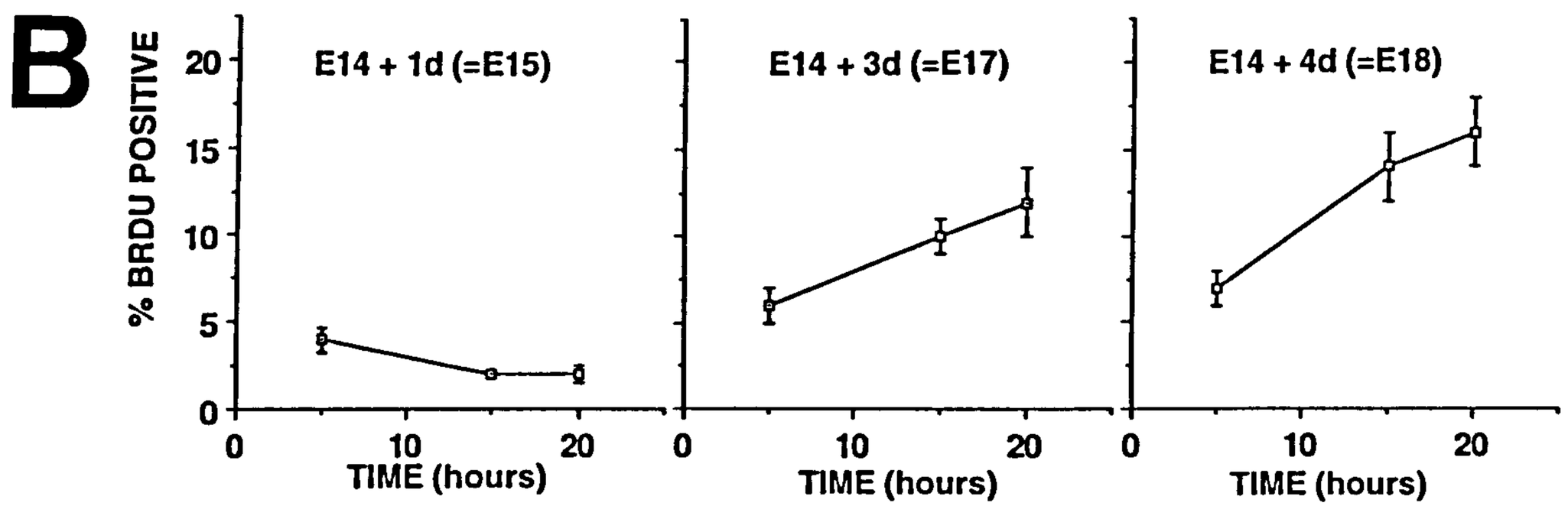
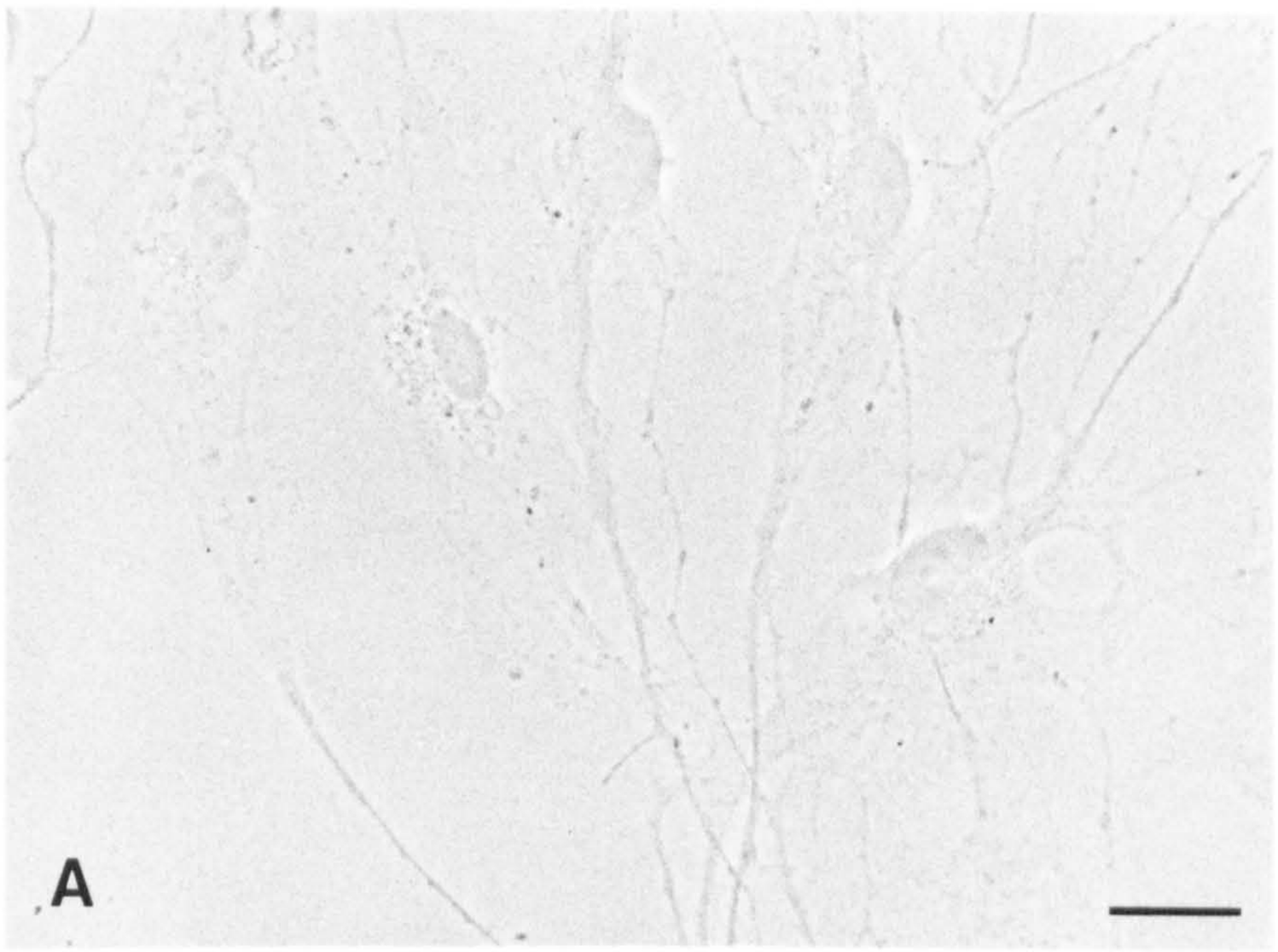

B
% BRDU POSITIVE
0
5
10
15
20
E14 + 1d (=E15)
E14 + 3d (=E17)
E14 + 4d (=E18)
0
10
20
TIME (hours)

Figure 6.6 Morphology of cells at 20 hr after replating following culture in NDFβ-2 for 4 days.

E14 cells cultured on LN coated tissue culture wells in 1.5ng/ml NDFβ-2, 1nM insulin and 100ng/ml IGF-1 for 4 days were replated and cultured for a further 20 hr in defined medium without NDFβ-2 but with 1nM insulin and 100ng/ml IGF-1 on LN coated glass coverslips. The cells were then stained for L1 expression. Some cells have a bipolar appearance but with flattened areas of cytoplasm around the nucleus. Other cells assume a mulitpolar morphology. (A) Phase contrast, (B) L1 expression. Bar = 20μm


A

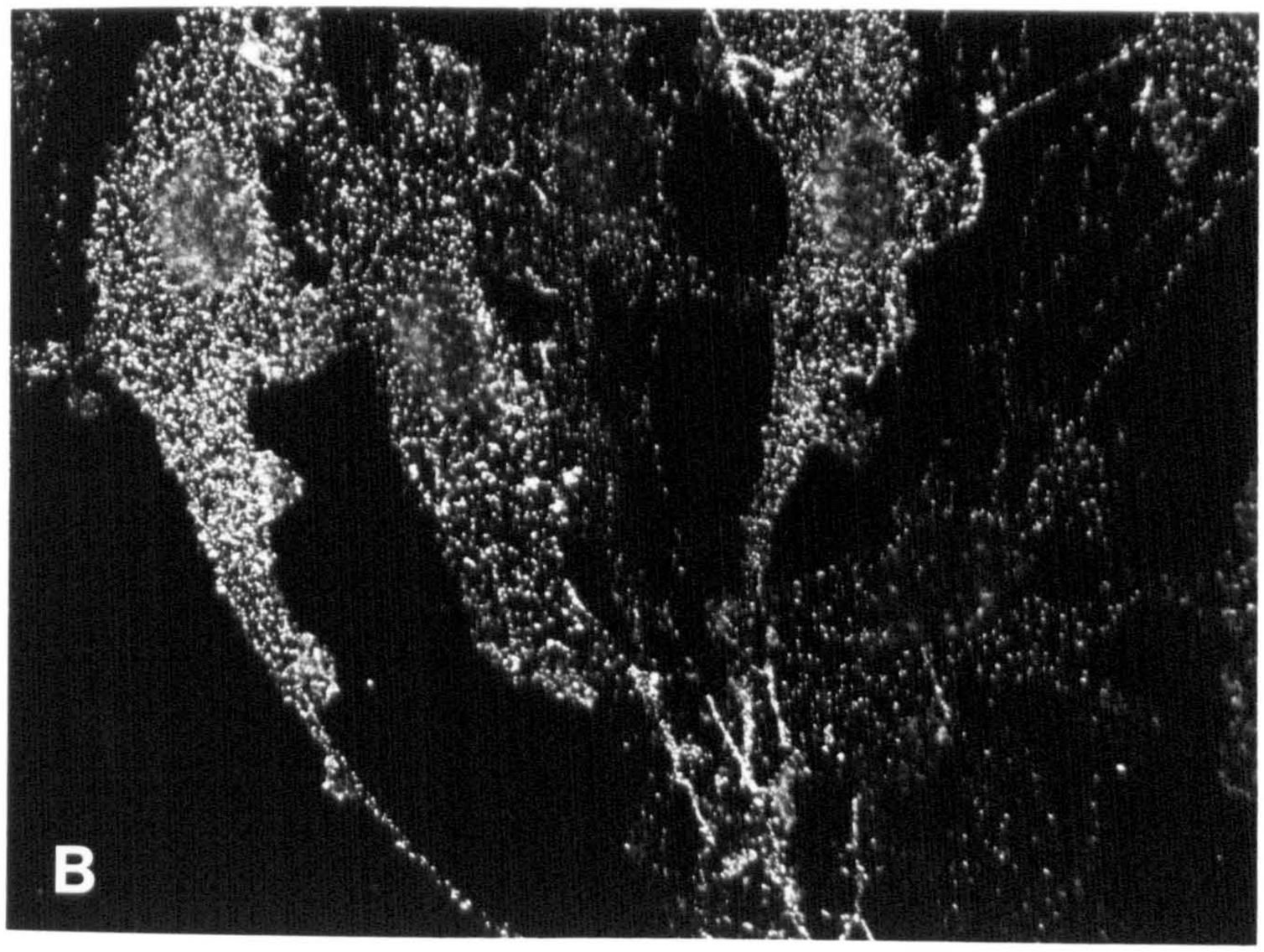
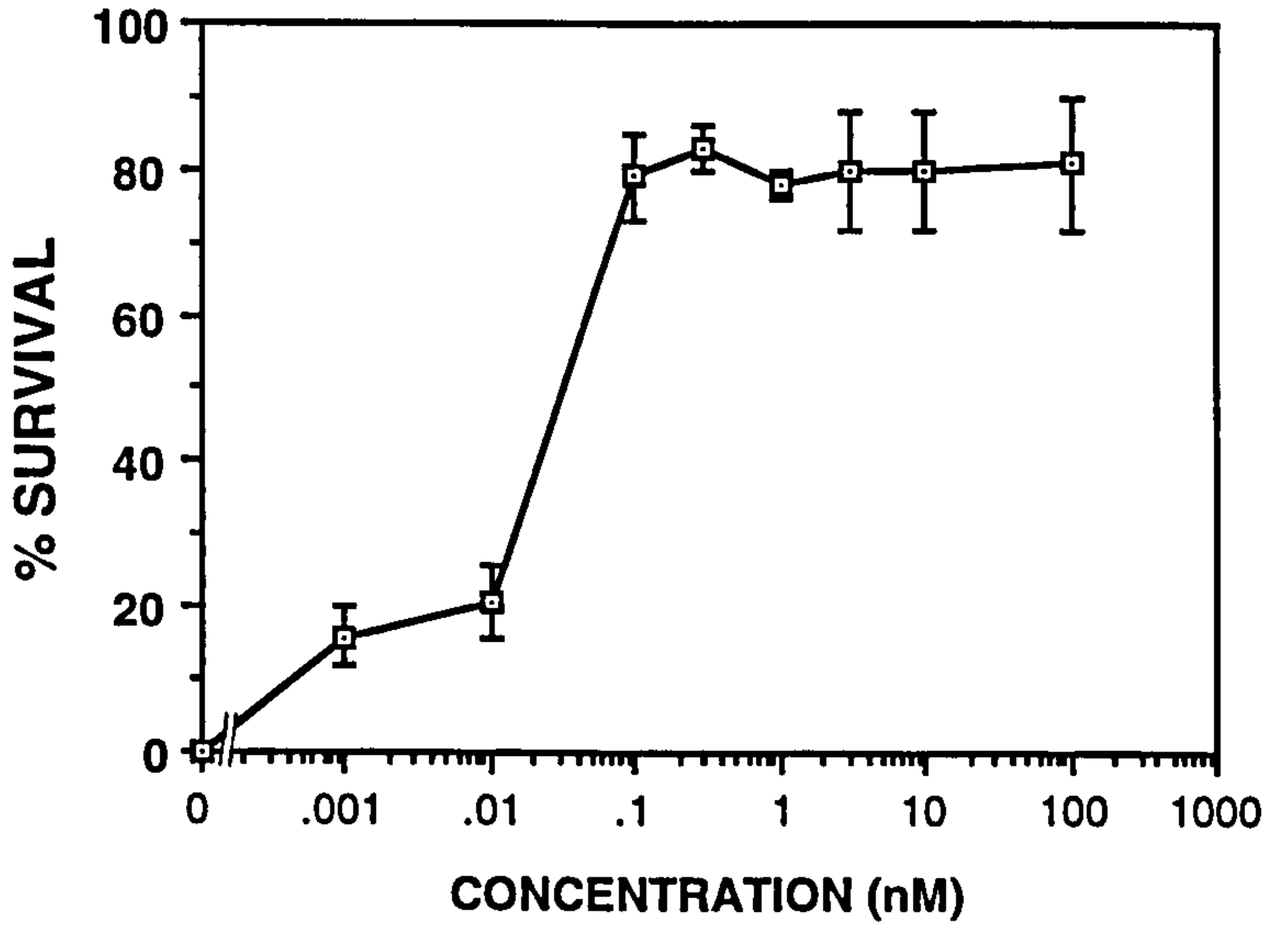

B

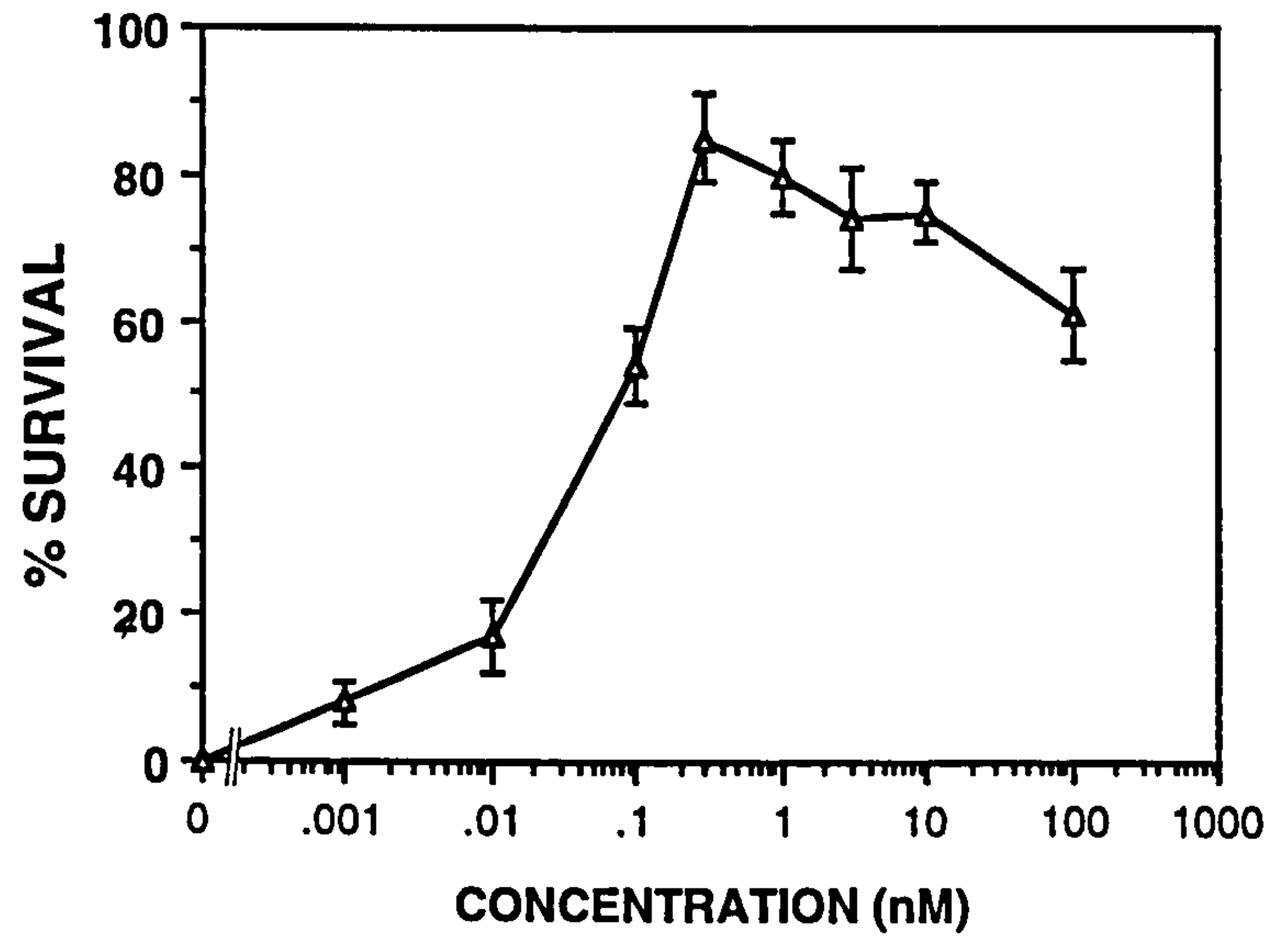
Figure 6.7 Endothelins 1, 2 and 3 promote survival of precursors in the presence of IGF-1

E14 cells were cultured in the presence of 1nM insulin and 100ng/ml IGF-1 with increasing concentrations of ET-1 (A), ET-2 (B) or ET-3 (C). At 20 hr the cultures were stained with L1 and survival calculated as described previously. All three ET isopeptides promote precursor survival in a dose-dependent manner and with similar affinities and effectivness. The points are an average of three experiments (A and B) or two experiments (C), error bars indicate SEM (A, B) or SD (C).

A


100
80
60
40
20
0
% SURVIVAL
0
.001
.01
.1
1
10
100
1000
CONCENTRATION (nM)

B


100
80
60
40
20
0
% SURVIVAL
0
.001
.01
.1
1
10
100
1000
CONCENTRATION (nM)

C

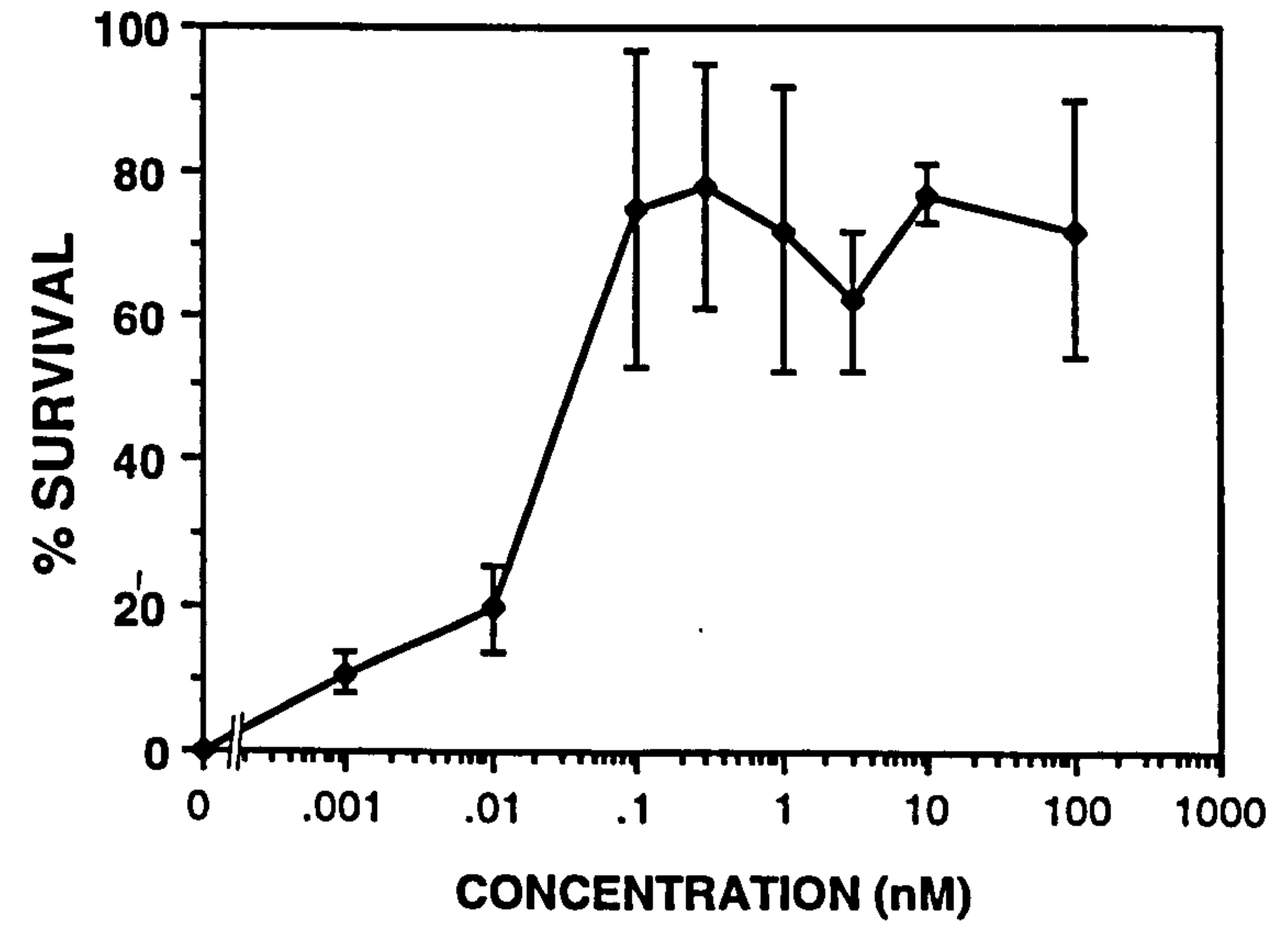
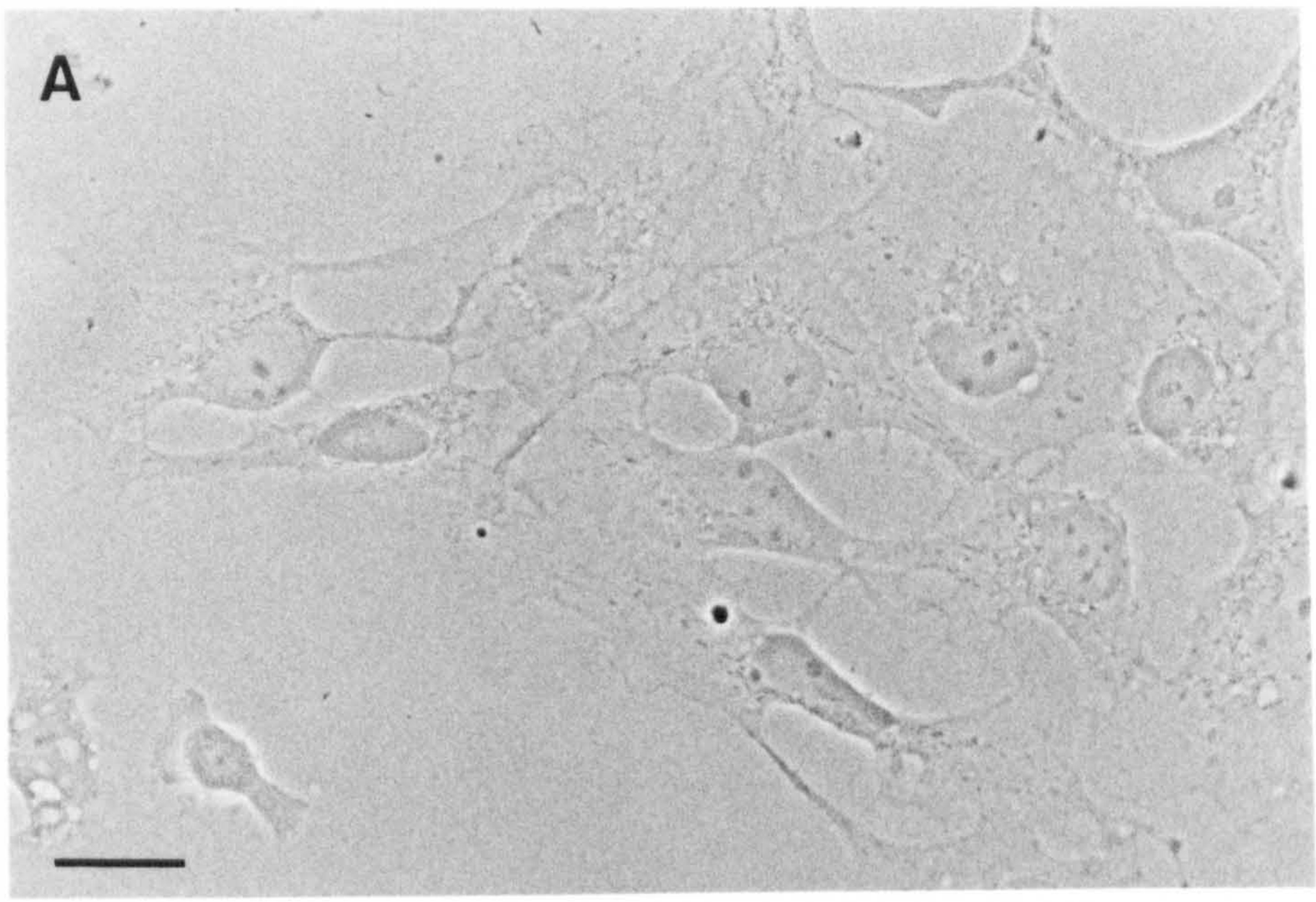

100
80
60
40
20
0
% SURVIVAL
0
.001
.01
.1
1
10
100
1000
CONCENTRATION (nM)

Figure 6.8 Morphology and L1 expression of E14 cells cultured in 0.3nM ET-1 for 20 hr.

E14 cells were cultured with 0.3nM ET-1, 1nM insulin and 100ng/ml IGF-1 for 20 hr and then stained for L1 expression. The morphology of the precursors in ET-1 at this time point is similar to that seen in NDFβ-2 after 20 hr in culture (Fig 6.1). (A) Phase contrast, (B) L1 expression. Bar = 20μm


A

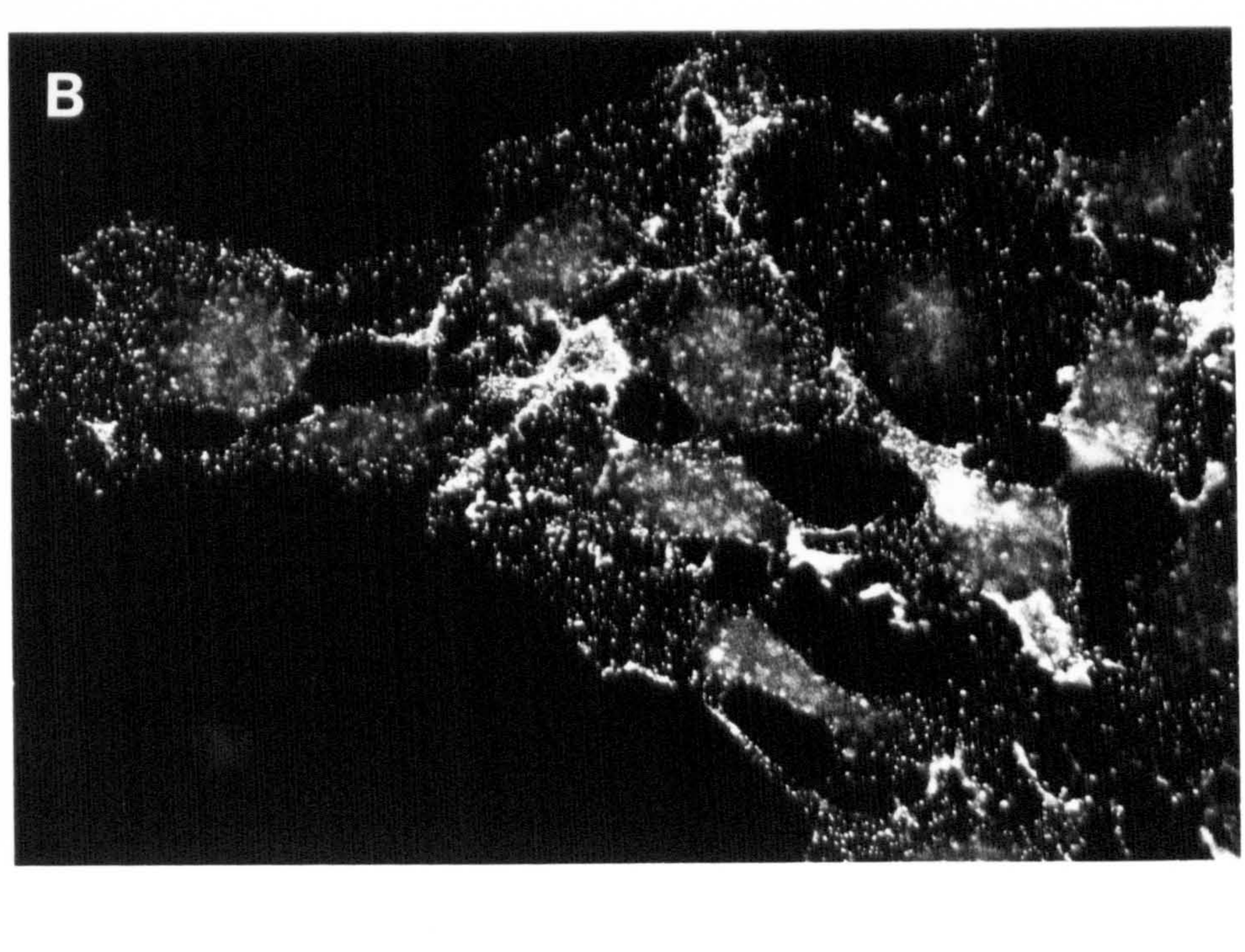

B

Figure 6.9 ET-1 promotes long term survival of precursors

E14 cells cultured in 0.3nM ET-1, 1nM insulin and 100ng/ml IGF-1 were stained for L1 at 20, 44, 68, and 92 hr after plating. The percentage survival was calculated relative to L1-positive cells that had attached and flattened 3 hr post-plating. ET-1 is able to prevent the death of 40% of the cells after 4 days in culture. Each point represents the average of two experiments, error bars indicate SD.

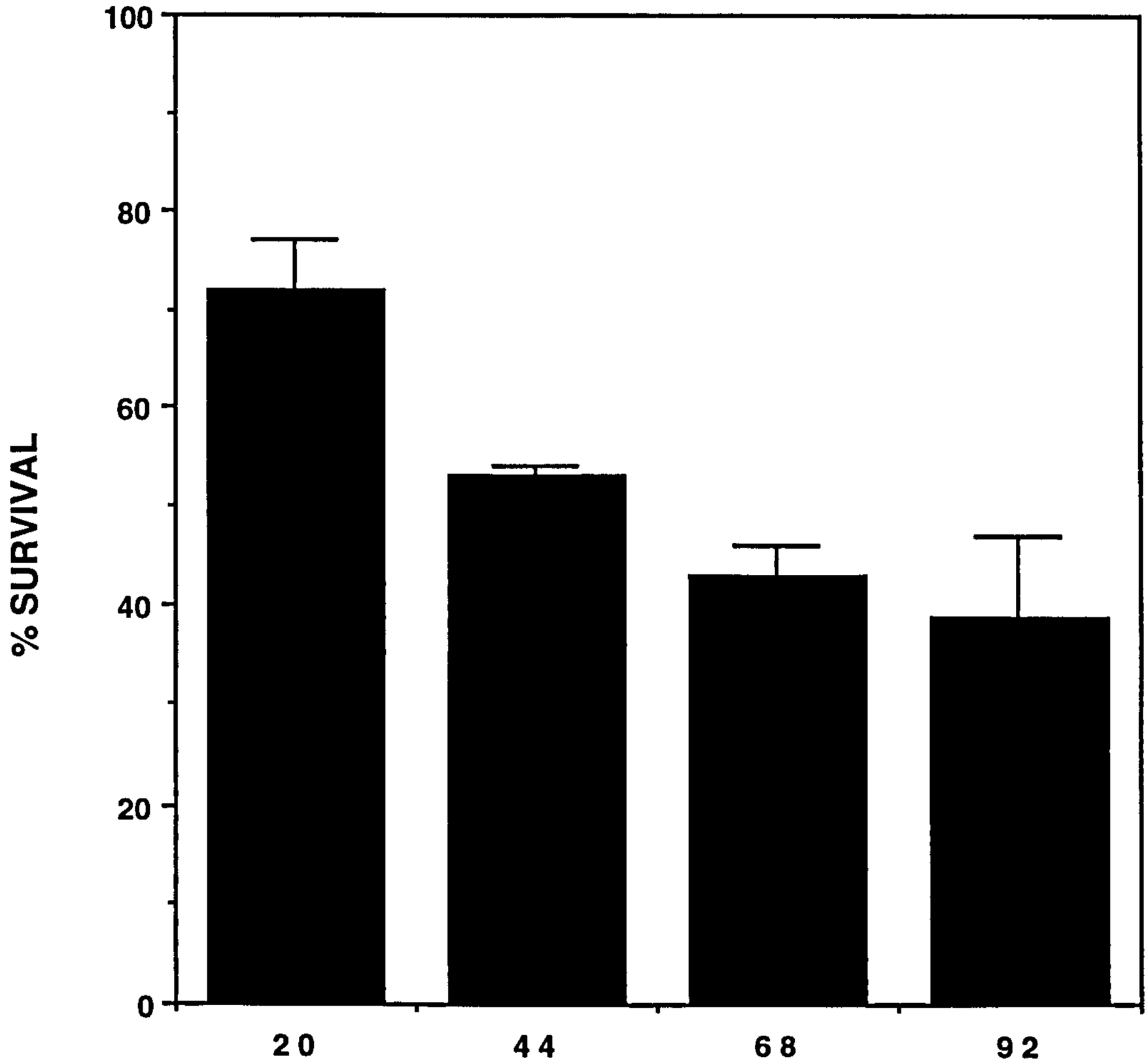
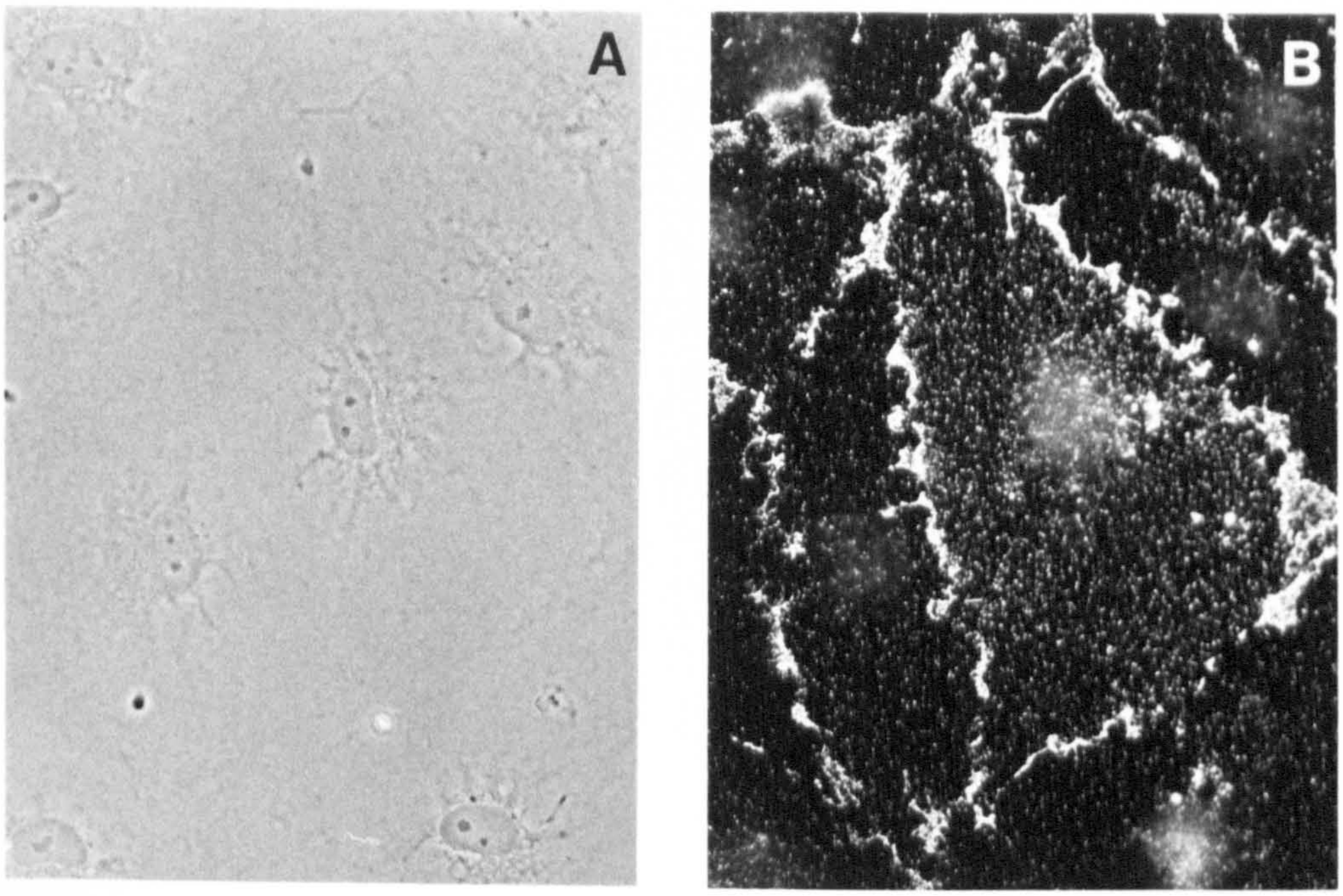

100
80
60
40
20
0
% SURVIVAL
20
44
68
92
TIME AFTER PLATING (hours)

Figure 6.10 Precursors cultured long term in NDFβ-2 or ET-1 with IGF-1

E14 precursors were cultured for 4 days with 1nM insulin and 100ng/ml IGF-1 and either 1ng/ml (40pM) NDFβ-2 (A, B), or 0.3nM ET-1 (C, D) and were then stained for L1 expression. (A) Cells cultured in NDFβ-2 have become extremely flattened during culture, the phase micrograph shows the nuclei of the cells depicted in (B). (B) Labelling the cells with L1 shows the extensive flattening of the cell cytoplasm after 4 days in culture with NDFβ-2. The brightest L1 staining is seen where adjacent cells are apposed. (C) Phase micrograph of precursors cultured for 4 days in the presence of ET-1. (D) The same cells labelled with L1. These cells have not flattened during culture; L1 expression is highest where adjacent cells overlap. Bar = 20μm.


A
B

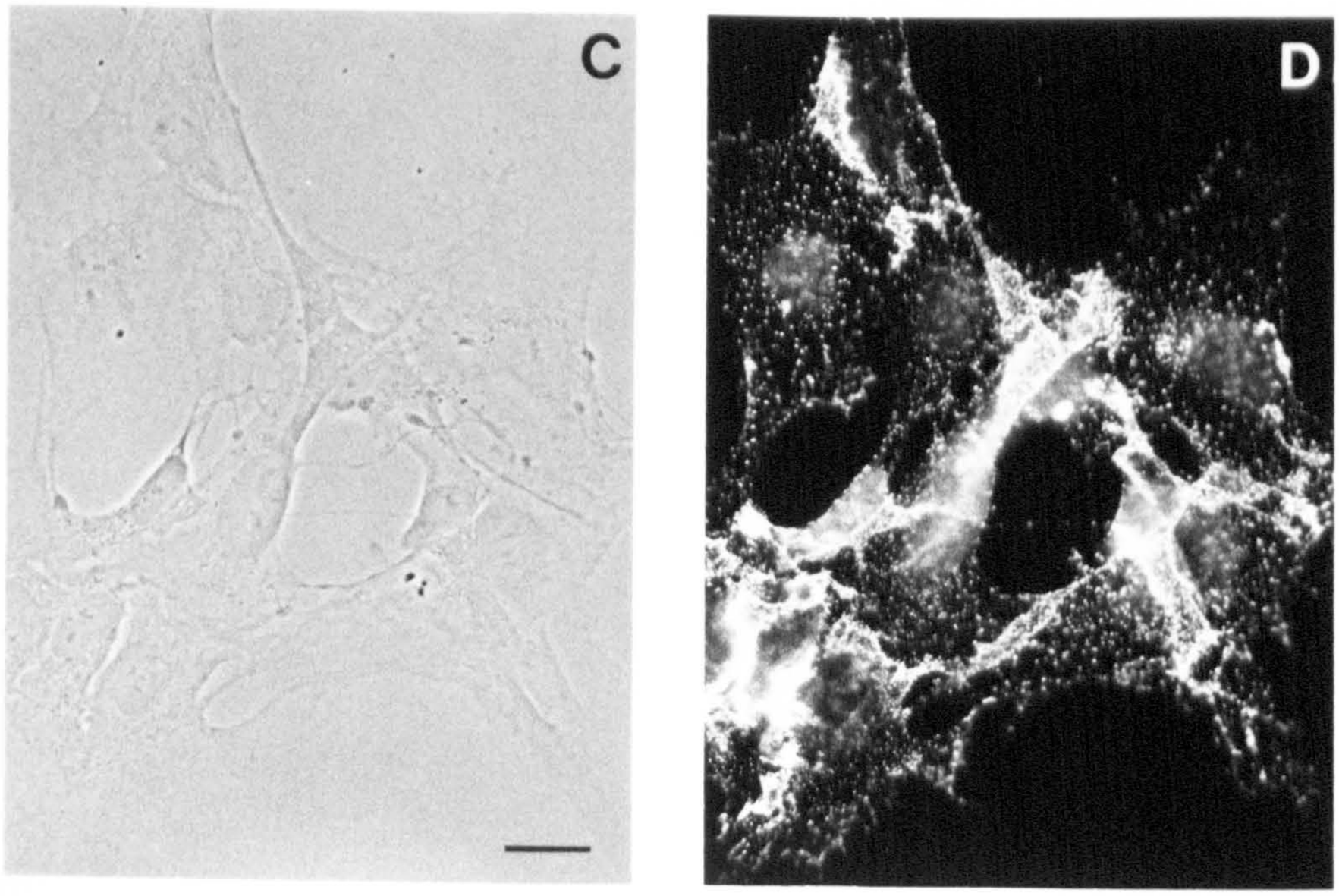
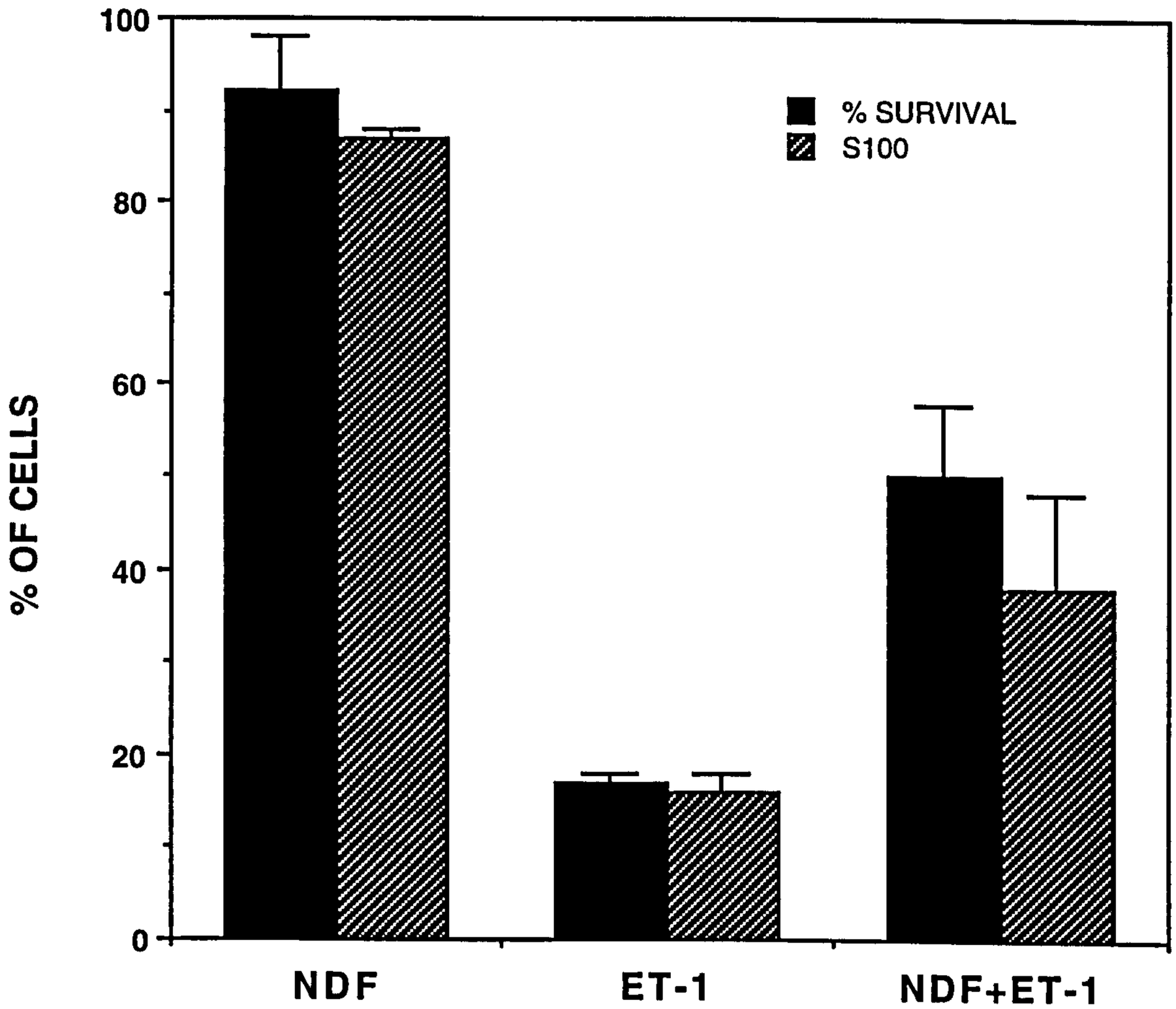

C
D

Figure 6.11 Comparison of survival and S100 expression by cells cultured for 4 days in NDFβ-2, ET-1 or NDFβ-2 with ET-1.

E14 cells were cultured in LN coated tissue culture wells with 1nM insulin, 100ng/ml IGF-1 and either 1.5ng/ml NDFβ-2, 0.3nM ET-1 or both 1.5ng/ml NDFβ-2 and 0.3nM ET-1. After 4 days the cells were replated onto LN coated glass coverslips and stained for L1 or S100 expression at 3 hr after plating, or cultured for a further 20 hr in 1nM insulin and 100ng/ml IGF-1 and assayed for survival by staining for L1 expression and comparing it to L1-positive cells adhered and flattened at 3 hr after replating. Cells replated from NDFβ-2-treated cultures have similar levels of survival and S100 expression to those of Schwann cells (Table 6.1); cells replated from ET-1-treated cultures have not matured towards the Schwann cell phenotype and have low levels of survival and S100 expression; culture with both ET-1 and NDFβ-2 produces cells with intermediate characteristics. The data represent the average of two experiments, error bars indicate SD.


100
80
60
40
20
0
% OF CELLS
% SURVIVAL
S100
NDF
ET-1
NDF+ET-1

DISCUSSION

Schwann cell precursors are able to survive and mature *in vivo*, presumably in response to signals present in the developing nerve. These signals are most likely to be of axonal origin and neurone conditioned medium can mimic the effects of axons in supporting precursor survival. The previous Chapter described the short-term effects of FGFs and IGFs but other factors must be present *in vivo* to promote long term survival of these cells. NDFs are likely candidates for neuronal survival factors, being present in appropriate neurones at the right time (Meyer and Birchmeier, 1994) and affecting the survival *in vivo* of cells early in the Schwann cell lineage (Meyer and Birchmeier, 1995). Here, the NDFβ-2 isoform has been used to investigate the long term survival and maturation of the Schwann cell precursors *in vitro*. Another growth factor family, the endothelins also support long term survival. Unlike NDFβ-2, endothelins have not previously been reported as survival factors for any cell type and, as far as is known, this is the first description of a member of the endothelin family affecting cells of the Schwann cell lineage.

NDFβ-2 promotes Schwann cell precursor survival

The NDFβ-2 protein used in this study is a non-glycosylated recombinant protein that corresponds to the extracellular sequence of this isoform. This NDFβ-2, in the presence of 1nM insulin and 100ng/ml (13nM) IGF-1, acts as a survival factor for precursors in a dose-dependent manner (Fig 6.2). The number of cells surviving at 20 hr in the presence of the highest concentrations of NDFβ-2 was higher than the number that had attached and flattened at 3 hr, indicating that this factor was mitogenic for these cells in short-term culture. This mitogenic effect has been studied further by Z. Dong (Dong et al., 1995) and is dose-dependent.

The work here was performed with NDFβ-2 in the presence of 1nM insulin and 100ng/ml (13nM) IGF-1, but Z. Dong has shown that NDFβ-2 will promote the survival of precursors in the absence of IGF-1, albeit at higher doses of NDFβ-2 (Dong et al., 1995), unlike the surivival induced by FGFs where IGFs are essential for survival. This suggests that binding of NDFβ-2 to its receptor(s) produces a response

in precursors that is sufficient to prevent death of these cells and does not require the activation of the IGF receptors.

The presence of NDFβ-2 promoted the long term survival of the precursors, with cells surviving 4 days *in vitro* (Fig 6.3). Again there was evidence for the mitogenic effect of this growth factor since the total number of precursors increased over the 4 day assay. It was notable, however, that this factor appeared to have survival and mitogenic properties that were specific for the precursors; the contaminating L1 negative cells did not increase with time in culture.

The ability of NDFβ-2 to prevent death of the precursors in a long term assay is similar to that of NCM (Jessen et al., 1994). When NCM is incubated with a soluble protein containing the ligand binding domain of ErbB-4 and then used in a survival assay, precursor survival decreases with increasing concentrations of ErbB-4. The action of NDFβ-2 can also be blocked in this manner but not the effect of FGF and forskolin, suggesting that ErbB-4 is blocking the action of NDFs present in the NCM (A. Brennan, data not shown; Dong et al., 1995). Even at the highest doses of ErbB-4, however, there is 20-25% survival, suggesting that other growth factors may be present in NCM that affect precursor survival (Dong et al., 1995).

Both Schwann cell precursors and Schwann cells express ErbB-2, ErbB-3 and ErbB-4 receptors (Cohen et al., 1992; Jin et al., 1993; Dong et al., 1995; Meyer and Birchmeier, 1995) and are able to respond to members of the NDF family. It has long been known that GGF, a member of this family, is mitogenic for Schwann cells *in vitro* (Lemke and Brockes, 1984, Marchionni et al., 1993; Minghetti et al., 1996). NDFs are produced by both sensory and motor neurones, and are detectable from early in development, from E10 in the mouse (~E11 rat) (Marchionni et al., 1993; Orr-Urtreger et al., 1993; Meyer and Birchmeier, 1994; Dong et al., 1995; Ho et al., 1995). Thus cells early in the Schwann cell lineage are exposed to these factors and have receptors for them. In mutant mice, where the active EGF-like domain of NDF has been deleted, the number of Schwann cell precursors observed in the developing peripheral nerves is greatly decreased (Meyer and Birchmeier, 1995). This may be a direct result of loss of NDF as a survival factor for these cells, or it may be as a result of earlier influences of the NDFs on neural crest development. GGF has been shown to direct neural crest development towards a glial fate (Shah et al., 1994). Thus the

absence of this factor may decrease the number of neural crest cells adopting a glial fate. Furthermore, since some Schwann cell precursors are still detectable within the mutant mice it is likely that other factors can partially replace NDFs as survival factors at this early stage.

NDFβ-2 generates Schwann cells from precursors *in vitro*

In Chapter 3, the developmental regulation of S100 expression and survival of precursors in defined medium was described. The transition from precursor to Schwann cell phenotype is rapid, taking place between E15 and E17 *in vivo*. Cells taken from E14 and E15 nerves and cultured in the presence of NDFβ-2 acquired the ability to survive in defined medium and S100 expression with a time course that paralleled that seen *in vivo* (Table 6.1). A rapid transition was seen between cells of E14 + 1 (=E15) and E14 + 3 (=E17) from low to high levels of survival and S100 expression. There is a slight discrepancy in the level of survival at E14 + 2 compared to that seen in the nerve at E16: while only 16% of cells survived in defined medium when plated directly from the nerve, 30% of E14 cells exposed to NDFβ-2 for 2 days survived. By E14 + 4 (=E18) the majority of cells expressed S100 and survived culture in defined medium at similar levels to that seen in cells directly from the nerves. When Schwann cells from newborn nerves are cultured for 1 day prior to replating it was found that 80% survived the procedure, while 100% of cells survived when plated directly after dissociation from the nerve. This is likely to be due to the nature of the replating procedure: when cells are dissociated from the nerve, the enzymatic and mechanical treatment is fairly harsh and cells that are not going to survive do not attach and flatten by 3 hr. However, the replating procedure is somewhat milder and could allow cells to attach that may be damaged in some way, thus producing a high 3 hr point which is then used to calculate survival of the cells.

A pilot study on E14 cells cultured for 4 days with 10ng/ml (400pM) NDFβ-2, in the presence of 1nM insulin but no IGF-1, produced similar levels of survival and S100 expression as that described with IGF-1 present (data not shown) showing that NDFβ-2 alone was capable of promoting the maturation of the cells.

These results, although suggestive of the precursors acquiring some Schwann cell characteristics in culture, do not show that the cells generated by NDFβ-2 can act like

Schwann cells. The test for this was the response of the cells to the Schwann cell mitogenic combination of bFGF and forskolin over 20 hr. Cells from E14 nerves cultured for 1 day in NDFβ-2 did not respond to bFGF and forskolin, but after a further 2 days *in vitro* with NDFβ-2 the cells showed an increase in DNA synthesis in response to these factors (Fig 6.5 B).

These results show that NDFβ-2 can promote the maturation of the precursors with a time course similar to that seen *in vivo*. That more than one developmental parameter is affected would suggest that NDFβ-2 can act on different genes and coordinate at the molecular level the change from precursor to Schwann cell.

One of the major differences between the precursors and Schwann cells is their morphology *in vitro*. Maturation of the precursors *in vitro* with NDFβ-2 did not produce this change in morphology without replating (not shown). After replating, many cells did adopt a morphology more closely resembling that of Schwann cells (Fig 6.6) but this transition was incomplete, with flattened sheets of cytoplasm extending from the cells in the region of the nucleus. It is possible that in the nerve, other factors are present that influence the morphology of the cells during this time of transition.

Endothelins promote Schwann cell precursor survival

The ETs used in this study are recombinant peptides corresponding to the mature ET sequences. All three ETs produced dose-dependent survival with maximal survival of 80% of precursors at 20 hr (Fig 6.7 A, B, C). The presence of 100ng/ml IGF-1 was required for the action of the endothelins suggesting that, like FGFs, the the activation of the type 1 IGF receptor was required for precursor survival and that activation of the ET receptors alone was insufficient to suppress precursor death. Other culture systems, particularly fibroblast cell lines, have shown that ETs require the presence of additional growth factors for mitogenic activity, and both high concentrations of insulin and IGF-1 are effective as co-mitogens (reviewed in Battistini et al, 1993).

The ET receptor involved in precursor survival is likely to be the ET_B receptor which, as described in the introduction, exhibits no selectivity for ETs 1-3. However, the antagonist studies failed to confirm this. BQ-123 produced no effect on the survival of precursors in ET-1 suggesting that the ET_A receptor is not involved. The ET_B

antagonist used, IRL 1038 has been reported to be highly selective for the ET_B receptor (Urade et al., 1992) but also failed to reduce the level of survival seen in the presence of ET-1. Since this study was undertaken it has become apparent that the activity of this antagonist is highly batch-dependent and that results obtained with this compound should be treated with caution (Bax and Saxena, 1994). However, two batches of IRL 1038 from two different sources were tried in this study and exhibited a similar lack of effect. It is possible that this compound cannot inhibit the ET_B receptors on the precursors, since an alternative transcript for ET_B has been found in rat brain that has tissue specific expression (Cheng et al., 1993). As described in the introduction, the ET_B receptors from different tissues display distinct responses to antagonists; for example, the compound RES701 inhibits ET_{B1} receptor-mediated vasorelaxation, while SB209670 inhibits ET_{B2} receptor-mediated vasoconstriction (Douglas et al., 1994). Further studies with the newer antagonists could clarify the receptor status of the precursors.

Schwann cell precursor maturation *in vitro*

The role of growth factors in cell differentiation and maturation appears to be complex, with some systems requiring a particular growth factor to direct cells towards a particular lineage, and other systems that require growth factors for survival and proliferation, with differentiation being cell autonomous (Shah et al., 1994). As described in Chapter 1, use of *bcl*-2 to prevent apoptosis in growth factor-deprived haemopoietic cells has shown that these haemopoietic cells will automatically differentiate into all cell types normally produced from the multipotent bone marrow (Fairbairn et al., 1993). In the haemopoietic system, therefore, it appears that the ability to differentiate is growth factor-independent and the presence of growth factors control the relative numbers of the cell types produced. Similarly, in the oligodendrocyte system, progenitor oligodendrocytes will differentiate into mature oligodendrocytes *in vitro* in the absence of growth factors (Raff et al., 1983). Thus it had been assumed that by preventing precursor death for several days the precursors would be able to mature into Schwann cells, as found in NDFβ-2. Therefore, the lack of maturation of the precursors after long term culture in ET-1 was surprising.

GGF is implicated in directing neural crest differentiation towards the Schwann cell lineage by promoting gliogenesis and suppressing neurogenesis (Shah et al., 1994). In

this light, it may be that the transition of precursors into Schwann cells requires NDF. Combining ET-1 and NDFβ-2 in long term cultures of precursors produced an intermediate population of cells, where ~50% expressed S100 and a similar number survived *in vitro* in defined medium for 20 hr (Fig 6.11). This result shows that ET acts to block the maturation effect of NDFβ-2. Similarly, NDFβ-2 appears to be driving precursor maturation towards the Schwann cell; if it was merely providing favourable conditions for precursor survival then there would be no attenuation of the maturation-blocking effect of ET-1.

As mentioned in the introduction, ETs are found in neurones of the spinal cord and DRG of adult human (Giaid et al., 1989) but as yet no description of their distribution in embryonic rats has been given. Thus it is not known whether precursors would be exposed to these growth factors *in vivo*. However, since some cell derivatives of the neural crest are directly influenced by ETs *in vivo* (Kurihara et al., 1994; Greenstein Baynash et al., 1994; Hosada et al., 1994) ETs must be present from early development onward, thus they could play a role in controlling development of the Schwann cell precursor lineage.

Control of Schwann cell precursor maturation does not use the same timing mechanism as oligodendrocyte differentiation.

The maturation of the precursors into Schwann cells does not exhibit the same elements of control as the maturation of oligodendrocyte progenitors. Oligodendrocyte differentiation it thought to be controlled by an intrinsic clock mechanism, where progenitors divide a set number of times before differentiating into oligodendrocytes regardless of whether mitogens are present (Raff et al., 1985). This clock can be controlled by thyroid hormones, glucocorticoids and retinoic acid (Barres et al., 1994).

ET-1 can promote survival of the precursors in long term cultures (Fig 6.9) but unlike NDFβ-2, the survival was not accompanied by appreciable DNA synthesis. If the Schwann cell precursors used the same mechanism of counting cell divisions prior to differentiation then the low cell division could account for the lack of effect of this growth factor on precursor maturation. However, long term culture in the presence of NDFβ-2 at low concentrations that generated DNA synthesis in only 40% of cells,

resulted in 78% survival at 20 hr in defined medium after replating, indicating that cell division is not a prerequisite for precursor maturation. Thus a clock mechanism that counts cell divisions does not determine the precursor to Schwann cell transition.

In summary, both NDFβ-2 and ET-1 can support long term survival of Schwann cell precursors *in vitro* in the presence of IGF-1, but only NDFβ-2 promotes the maturation of precursors to Schwann cells. ET-1 blocks precursor maturation, but this can be partially overcome by NDFβ-2, showing that NDFβ-2 actively drives precursors to the Schwann cell phenotype. The maturation does not require the cells to undergo division, suggesting that the mechanism for the timing of maturation is not the same as that found in oligodendrocyte development.

CHAPTER 7

GENERAL DISCUSSION

The aim of this work was to study in detail the glial cells of the peripheral nerves in the rat embryo. This has been approached in three ways: firstly, a study has been made of some of the phenotypic differences between glial cells of early embryonic nerves, the Schwann cell precursors, and the cells found earlier and later in development, namely the neural crest and Schwann cells; secondly, the nature of precursor death *in vitro* and the survival requirements of these cells, has been explored; and thirdly, the maturation of these cells into Schwann cells *in vitro* has been investigated.

The early development of nerves has previously been studied with regard to axonal outgrowth and innervation of targets but, prior to this work, the glial cells associated with these nerves had not been studied in any depth. This study has shown that these cells are not neural crest cells although they are morphologically similar *in vitro*, but, like cultured neural crest cells, retain the survival response to members of the FGF family of growth factors in the presence of a member of the insulin growth factor family (Bannerman and Pleasure, 1993). However, the precursors exhibit an altered response to other factors, with NDFs or ETs together with IGF-1 also being able to promote survival in precursors but not in neural crest cells. NDFs have been reported not to be mitogens for neural crest cells, adding to the differences between the two cell types (Shah et al., 1994). In addition, a change is seen in the expression of the phosphoprotein GAP-43, which is absent in neural crest but expressed by all precursors.

This study has also established that the Schwann cell precursors express many proteins found on Schwann cells of the perinatal and adult nerve, with the phenotype more closely resembling that of the non-myelin-forming Schwann cells than that of myelin-forming Schwann cells (Table 3.3). However, the precursors exhibit four major differences from Schwann cells, namely morphology, lack of expression of the Ca^{2+}-binding protein S100, an inability to survive culture in defined medium and an inability to increase DNA synthesis in response to the Schwann cell mitogenic combination of FGF, IGF and forskolin. Thus the glial cells found in E14 and E15

nerves are not Schwann cells, and can be considered a distinct cell type in the Schwann cell lineage.

The transition of precursors into cells with the Schwann cell phenotype is rapid, occuring essentially over one developmental day at E16. Expression of S100 correlates with survival in defined conditions suggesting that these two features may be linked. Exogenous S100, however, does not act as a survival factor for precursors, and in studies where cells were stained for S100 at 20 hr after replating, some surviving cells did not express this protein (data not shown). Although the exact nature of the S100 protein function is unknown, the ubiquitous expression in all PNS glia of perinatal and adult rats would suggest that it has an important role in glial function. It is possible that precursor S100 can function as trophic support for neurones; exogenous S100 as has been reported to increase the survival of motor neurones in the developing chick (Bhattacharyya et al., 1992). The onset of S100 expression coincides with the end of the period of motor neurone death, since the rate of neuronal loss starts to decrease at E16 in mouse (equivalent of E17 rat) (Lance-Jones, 1982). Schwann cell precursors are believed to be a source of GDNF that is trophic for developing motor neurones (Henderson et al., 1994). Thus, a mutual support system may function in the developing nerve.

The ability of NCM (Chapter 3 and Jessen et al., 1994) and neurones in co-culture systems (Roufa et al., 1986; Smith-Thomas et al., 1990; Dong et al., 1995) to promote precursor survival and maturation strongly suggests that neurones are the source of factors necessary for precursor survival. These cells are in intimate association with neuronal membranes from the earliest times of axonal outgrowth (Dahm and Landmesser, 1988; Carpenter and Hollyday, 1992a,b; Jessen et al., 1994) and a reliance on neuronally-derived factors for survival and mitogenesis provides a mechanism for controlling the final Schwann cell numbers within the nerve. These studies have provided the first evidence that cells of the Schwann cell lineage can undergo apoptosis in the absence of survival factors.

A recent paper, describing studies of cell death *in vivo* during nerve development in the chick, has shown that glial cells in the nerve undergo two waves of cell death (Ciutat et al., 1996). Studying the glial cells of the ventral root, the first wave occurs at E5-6 with a second peak of death occuring at E8-9 coinciding with the peak time of

motor neurone death. Ciutat and collegues suggest that the first wave of glial cell death occurs at a time when the axons are grouped into fascicles and some Schwann cells may be unable to obtain sufficient survival factors because of their location on the outside of the nerve bundles. The work reported in this thesis suggests that the first period of natural cell death may correlate with a transition from neural crest cells to Schwann cell precursors. The transition of crest cells to precursors is accompanied by a change in growth factor requirement and, as described in Ciutat et al. (1996), the newly formed precursors may now be unable to survive without axonal contact where previously the neural crest cells were viable. Alternatively, the first wave of cell death may represent neural crest cells that have failed to make the transition to Schwann cells, and therefore cannot be supported by axonal growth factors at this stage.

Ciutat et al. (1996) also show that neuronally-derived factors are required to prevent the apoptosis of glial cells in the developing nerve. In the E7.5 chick embryo, Schwann cell precursors are seen to die in increased numbers when axonal degeneration is induced by killing motor neurones with high levels of N-methyl-D-aspartate (NMDA), or by killing motor and sensory neurones with β-bungarotoxin. This embryonic age in chick is developmentally equivalent to E14 in rat (Ciutat et al., 1996), suggesting that the death observed *in vitro* in rat Schwann cell precursors in the absence of neurones or NCM is not an artifact due to culturing the cells, and is likely to reflect the behaviour of precursors in the rat developing nerve *in vivo*.

The Schwann cell precursors are highly motile (Jessen et al., 1994) and apoptosis could be an important mechanism in controlling survival of these cells in inappropriate locations away from axonal contact. The timing of susceptibility of precursors to apoptosis in the absence of survival factors coincides with the period of natural cell death of neurones: from ~E12 to E19 in rat lumbar motor neurones (projecting data available from mouse) (Lance-Jones, 1982), and from E15 to birth in rat lumbar sensory neurones (Coggeshall et al., 1994).

Prior to the transition to Schwann cells, the precursors exhibit survival factor requirements that are quite specific. Three members of the FGF family can promote short-term survival in the presence of an IGF or high levels of insulin. In most cell systems, high levels (1μM) insulin can substitute for IGF-1 in activating the type-1 IGF receptor (Rechler and Nissley, 1985; Sara and Carlsson Skwirut, 1988).

However, the present study shows that insulin cannot elicit the same survival effects as IGF-1 unless there is elevation of cAMP. This may be due to a difference in affinity for insulin by the IGFBPs, but it may be that a novel form of the type 1 IGF receptor exists in the early embryonic nerve, as is suggested from the studies of mice deficient in IGFs and their receptors. These mutation studies suggest that an alternative IGF receptor exists during development, but the evidence points to this receptor preferentially binding IGF-2. However, without isolation of the protein no definite comment on binding properties can be made (Baker et al., 1993; Liu et al., 1993).

The short-term effect of the FGFs on blocking apoptosis in precursors suggests that they are unlikely to play the main role in precursor survival *in vivo*. The discovery that NDFβ-2 not only promotes long-term survival but also stimulates DNA synthesis, makes this factor, or one of the members of the NDF family, a likely candidate for an *in vivo* survival factor and neuronally-produced mitogen (Dong et al., 1995; Morrisey et al., 1995). NDFs are present in the developing nerves at appropriate ages to support survival, and both neural crest and cells of the Schwann cell lineage express receptors for these factors making it highly probable that they play a vital role in Schwann cell development (see above, and Chapter 6 for references). As described earlier, precursors are not entirely absent from early nerves of mice lacking the active domain of NDF (Meyer and Birchmeier, 1995), indicating that other growth factors may be present in the nerve that can act as survival factors in the absence of NDF. The discovery that ET-1 also has long-term survival effects on the precursors raises the possibility that this could act as such a factor. As yet the embryonic distribution of the ETs is not known and work is underway to determine whether they are present in the developing PNS.

Perhaps the most surprising data from this study is that NDFβ-2 can promote the maturation of precursors to cells of a Schwann cell phenotype with a time course not unlike that seen *in vivo*. The presence of this single growth factor appears to be sufficient to elicit the multiplicity of changes that occur during this transition from precursor to Schwann cell. Some of the changes have been described here, but it is certain that many others have yet to be elucidated. For example, the timing of transition coincides with strong elevation of expression of the zinc-finger transcription factor Krox 20. Expression of the factor appears along the entire length of peripheral

nerve at around E15 in the mouse (equivalent to rat E16-17) (Topilko et al., 1994). The function of Krox 20 at this age is unknown, but ablation of the gene blocks PNS myelination at an early stage, suggesting that its expression in the nerve determines full maturation of the myelin-forming Schwann cells.

How NDF acts to promote the maturation of precursors to Schwann cells is not known. It may be that it acts purely to suppress apoptosis. In this case, differentiation of Schwann cells would be due to an intrinsic programme that requires no additional signals for its completion. If such a programme exists in Schwann cells, then blocking cell death would be sufficient to allow differentiation of precursors; such an event may be considered a default pathway. Alternatively, NDF may not only block cell death but also drive the differentiation programme. At present, it is not clear which of these explanations best describes the role of NDF in Schwann cell maturation.

It is of interest that the entire population of Schwann cell precursors in the nerve apparently undergo transition to Schwann cells in an approximately synchronous fashion. As mentioned in Chapter 6, the control of this developmental change is not due to a clock counting cell division, as is the case for oligodendrocytes. The control of maturation exerted on the precursors by NDF does not require cell division, a factor that might permit the change to Schwann cells to occur over a relatively short developmental period. When the transition occurs *in vivo*, it would be possible to look to other events in nerve development, such as the initiation of electrical activity, for a signal to coordinate the change. However, *in vitro* there is no such change of external signals, which raises the question of the nature of the switch that controls maturation. The cultures used in these experiments have two drawbacks i) they are not 100% pure, and ii) precursor cell numbers and density increase during the course of the experiment, both raising the possibility that cell-cell signalling might take place in these cultures. Factors produced by a small number of cells that have attained maturation to Schwann cells may act in a paracrine manner on surrounding cells to produce an apparently synchronous switch of phenotype. To determine whether the survival and maturation of Schwann cell precursors requires endogenously produced autocrine factors as well as NDFs, experiments with cultures at clonal density (50 cells per 13mm diameter coverslip) are planned.

The NDFs seem to act as truly multifunctional growth factors in PNS glial development from the very earliest stages of neural crest development to the maturation of Schwann cells in the nerve. As mentioned in Chapter 1, the models proposed for growth factor effects on cell fate determination are that i) growth factors present in the environment determine the developmental choice of the cell, or ii) that the cell makes a choice stochastically, with growth factors acting to amplify this decision, selectively promoting survival and/or proliferation of a differentiated cell type. It is difficult to say with certainty which model represents the generation of glia in the PNS. Among the first known actions of NDF on neural crest is suppression of the neuronal transcription factor MASH 1 (Shah et al., 1994) and in this way NDF may direct crest cells towards a glial fate. It is not known whether neurones arise by stochastic events in the neural crest. However, the first neuroblasts can act as a source of NDFs to direct differentiation of other neural crest cells away from the neuronal fate. As desribed in Chapter 1, the earliest neuroblasts and glia in the developing sympathetic ganglia appear to have complex cell-cell interactions, with NDF production from the neuroblasts enhancing NT-3 production by the glia, which in turn promotes survival of the neuroblasts, followed by trkA expression and their subsequent transition from NT-3-dependent cells to NGF dependent cells (Verdi et al., 1996).

Other growth factors are likely to be produced by the developing neuroblasts that act as survival and proliferation factors for the glial-determined neural crest cells since NDF alone does not seem to promote survival of these cells (Chapter 3) and is not a mitogen for them (Shah et al., 1994). Once cells have started to develop as glia, the NDFs act as trophic and mitogenic factors as shown in the present work, eventually controlling the timing of maturation of glial cells in the nerve into Schwann cells. It will be interesting to determine the mechanisms behind this sequence of events.

A role for endothelins in peripheral nerve development remains hypothetical at this stage. That they are present in neurones of the adult DRG suggests that the glial cells are exposed to them, but whether they are available to influence cells in the developing nerve is not known. Similarly, it is not clear whether precursors cultured in ET-1 and IGF-1 would eventually acquire the Schwann cell phenotype, a few cells do seem to mature in the four day cultures, and it could be that this factor combination severely

delays maturation. It will be interesting to see whether cultures of precursors exposed for longer than four days to this growth factor combination have increased numbers of cells maturing. Other work planned is to look for other growth factors, such as FGFs, that can influence not only ET-1 effects but also those of NDF.

It is possible that ET-1 is altering the fate of the precursors; little work has been done to determine whether these cells can differentiate along other paths of neural crest development. In preliminary studies on precursors from pigmented rats, conditions reported to induce melanogenesis in avian neural crest cells and Schwann cells in culture (addition of bFGF, TPA and forskolin) (Stocker et al., 1991) failed to produce any cells that resemble melanocytes (A. Brennan, A. Sinanan and K. R. Jessen, unpublished data). This may be a species difference in the point of commitment to a particular lineage or a requirement for additional growth factors in rat melanogenesis, for example ETs. It is interesting to note that absence of ET-3 or the ET_B receptor results in an absence of melanocytes from the trunk region of mutant mice (Greenstein Baynash et al., 1994).

Future studies are planned to investigate PNS glial development in ET_B mutant mice. While overt differences in peripheral nerve development of these mice have not been reported, the effects of ETs implied from the current work may influence timing rather than any gross change in phenotype.

In summary, this study shows that the Schwann cell precursor is a distinct intermediate in the Schwann cell lineage, with transition to a Schwann cell phenotype occuring rapidly over one developmental day. NDFs can promote not only survival and proliferation of the precursor, but also maturation *in vitro* with a time course that is quite similar to that *in vivo*. Other growth factors, namely FGFs and ETs, may affect precursor differentiation, suggesting that a complex growth factor interplay might determine the timing of peripheral nerve development.

CHAPTER 8

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